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SECTION MEETINGS

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| **ILLINOIS** | |
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| University of Southern California | February 2, 1950 |

## Prevention of Diabetes on Forced Fed Rats Under Prolonged Diethylstilbestrol Administration. (17668)

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Ingle(1) observed the appearance of glycosuria in normal rats or its increase, if already present, in partially pancreatectomized rats, under prolonged administration of diethylstilbestrol. This diabetogenic effect took place in forced fed animals. Diethylstilbestrol had no effect on the glycemia or glycosuria of partially pancreatectomized male rats, providing the animals were allowed to eat *ad libitum*(2,3). Ingle believes that the reason of this failure is the diminished food intake due to the anorexia produced by that estrogen. In previous works(4,5) we showed that the estrogens had a protective and androgens an aggravating action on the onset and course of diabetes of partially pancreatectomized rats (surgical ablation of 95% of pancreatic mass) when feeding was *ad libitum.*

The present paper shows that, in forced fed rats, a protective action followed the initial—although not constant—diabetogenic action of diethylstilbestrol.

*Methods and material.* White rats from

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1. Ingle, D. J., *Endocrinology,* 1941, v29, 838.

2. Janes, R. G., and Dawson, H., *Endocrinology,* 1946, v38, 10.

3. Ingle, D. J., *J. Clin. Endocrin.,* 1947, v7, 449.

4. Foglia, V. G., Schuster, N., and Rodríguez, R. R., *Rev. Soc. argent. Biol.,* 1947, v23, 202; *Endocrinology,* 1947, v41, 428.

5. Lewis, J. T., Foglia, V. G., and Rodríguez, R. R., in press.

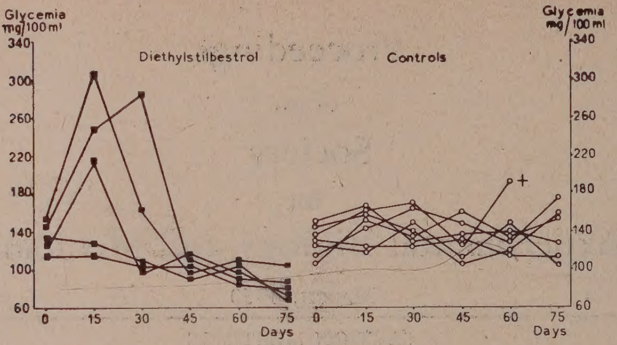

FIG. 1.

Variation of the glycemia of castrated and partially pancreatectomized female rats (ablation of 95% of the pancreas) under forced feeding, injected with 50 μg/day of diethylstilbestrol. Controls.

the Institute strain were used throughout the investigation. In the first experiments female rats of 130 to 150 g of body weight were castrated, followed a week later by removal of 95% of the pancreas using Foglia's technic(6). Ten days later, after recovery from the 2 surgical procedures, forced feeding was started. After 5 weeks under the special diet (Fig. 1) the entire group was divided in 2, the animals of the control group receiving 25 mg of lactose daily, subcutaneously, in 0.5 ml of 0.9% ClNa solution; the animals of the second group were injected daily with the same dose of lactose in the saline solution and with 5μg of diethylstilbestrol dipropionate in addition.

The diet used in the forced feeding had the following composition:

| | g |
|---|---|
| Cellu-flour | 120 |
| Salt mixture (McCollum-Davis) | 40 |
| Yeast | 100 |
| Dry milk | 600 |
| Wheat germ oil | 12 |
| Mazola oil | 220 |
| Cod liver oil | 12 |
| Water | 1400 |

The final % composition of the diet was:

| | g % |
|---|---|
| Water | 53.00 |
| Dry residue | 47.00 |
| Carbohydrate | 15.00 |
| Protein | 8.30 |
| Fat | 17.35 |
| Cellulose | 3.00 |
| Ash | 3.35 |

The animals received a total daily dose of 25 ml, divided in 2 rations. They were fed from 8 to 8:45 a.m. and from 4:30 to 5:30 p.m. The mixture was warmed up to 35°C immediately before feeding. It was observed that the animals remained during 2 or 3 hours in a state of sopor after the feeding. In order to avoid alimentary shock a total daily ration of 10 ml was given at the beginning, increasing it in 1 ml daily, in such a manner that 2 weeks later the animals were receiving the full daily dose of 25 ml. The animals were kept under such constant diet and without any treatment during the follow-

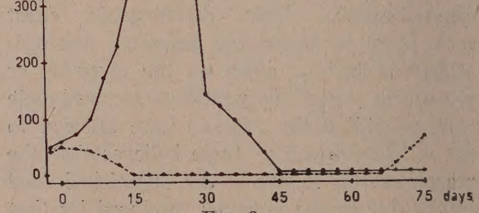

FIG. 2.

Variation of the glycosuria of castrated and partially pancreatectomized female rats (ablation of 95% of the pncreas) under forced feeding, injected with 50 μg/day of diethylstilbestol. Controls (average of 5 rats injected and 7 controls).

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6. Foglia, V. G., *Rev. Soc. argent. Biol.*, 1944, v20, 21.

ing 3 weeks, in order to allow the glycemia and glycosuria to reach a constant level.

Each animal was kept separately in a single cage, at a temperature from 22°C to 23°C. They drank water at will. The urine of each animal was collected in the usual manner, preserved under toluol and every 48 hours determination of its glucose content was made by the Benedict method. Blood samples were drawn from the tail 7 hours after the morning forced feeding and glycemia was determined by the Hagedorn and Jensen method.

*Results.* The data of Fig. 1 and 2 show that pancreatectomized rats, without hyperglycemia up to the moment when diethylstilbestrol at a 50 $\mu$g daily dose was started, showed an initial raise of the glycemia and glycosuria. This increase in the glycemia and glycosuria took place in most but not all of the animals. In the others the glycemia and glycosuria variations were only temporary as they dropped to their initial level approximately 1 month after the diethylstilbestrol treatment, and while still under it. One of the non-diabetic animals died, probably due to the toxic effect of the drug. At the moment when diabetes appeared in the control group, injected with lactose, glycosuria and blood sugar level were normal in all the diethylstilbestrol injected animals. Table I shows less increase of weight in the diethylstilbestrol injected animals as compared with the control series at the end of the experiment.

Autopsy findings (Table I) of the diethylstilbestrol group consisted in enlargement of the pituitary and adrenal glands.

*Histology.* The microscopic study of the pancreas was performed by Dr. A. F. Cardeza. Marked hyperplasia of the insular tissue was found in the diethylstilbestrol series (Fig. 3a). Confluent masses of newly formed islets could be observed of a polylobulated and ramified appearance; transformation of the centro-acinous cells in beta cells, in different stages of the process could be seen in some small islets. Bands of sclerosis connective tissue were seen in the periphery of some insular masses. At times, these bands penetrated and dissociated the beta cells trabecules. Other islets, clearly differentiated from the exocrine part did not show evidence of sclerosis; cytologically, the beta cells and the typical granules were the ones which almost exclusively could be demonstrated. The beta cells were hypertrophic, sometimes globulous or spherical, with a clearly visible perinuclear negative image of the Golgi apparatus when stained with Gomori's stain. The pancreas of the non-diabetic controls (Fig. 3b) showed foci of less intense hyperplasia of the insular tissue than the animals injected with diethylstilbestrol. Collagenous tissue was present around and within the islets. The granuli of most of the beta cells were well stained. The diabetic control rats showed islets with very scant hyperplasia of the insular tissue. The beta cells showed degenerative lesions with vacuolization of the cytoplasma, nuclear atrophy and pyknosis. The pituitary glands of the diethylstilbestrol-injected rats were enlarged, with sinusoid dilatations, blood lakes and necrobiotic zones. The cytology showed an equal number of cromphobic and basophilic cells, the latter with a large intraprotoplasmatic acidophilic vacuola. These elements were smaller than in the castration cells, the vacuolar content was more acidophilic and similar to the colloid substance. The pituitary gland of the control animals had the typical appearance of a castration gland, with hypertrophic basophilic cells, excentric nuclei, the cytoplasma occupied by a large vacuola and a decrease in the eosinophilic cells. Loss of hair as previously described(5) was prominent in the diethylstilbestrol injected rats.

TABLE I.

Pituitary and Adrenal Weight (mg % of Body Weight) of Castrated and 95% Pancreatectomized Female Rats Under Forced Feeding. Average of 6 Injected Animals and 7 Controls

| | Lactose | Diethylstilbestrol 50 $\mu$g/day |
|---|---|---|
| Hypophysis | 4.7 | 17.9 |
| Adrenals | 28.5 | 46.9 |
| Final body wt, g | 234 | 203 |

*Discussion.* The increase in glycosuria observed by Ingle after diethylstilbestrol administration persists for a variable period, usually less than a month and is accompanied by hyperglycemia, which follows the same chronological order. During this period, so

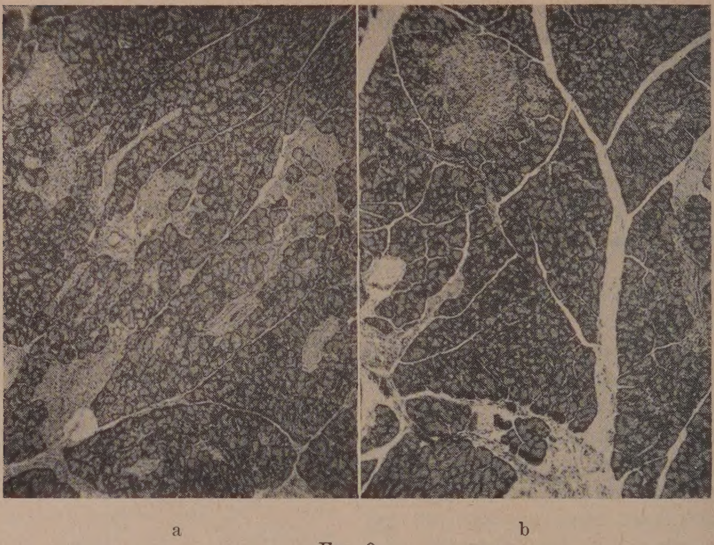

FIG. 3.
Pancreas of non-diabetic partially pancreatectomized rats (removal of 95% of the pancreas), under forced feeding, injected with: (a) diethylstilbestrol, showing marked hypertrophy and hyperplasia of the islets of Langerhans; (b) lactose, showing sclerosis inside and outside the islets. Hematoxylin-eosin. × 60.

animals, both with and without diabetes, died probably due to a toxic action of the drug. Some factors have been published which agree with our own findings. Ingle's Fig.(1) show that stilbestrol does not maintain the increase in glycosuria initially produced by it in normal and pancreatectomized rats. The same author(7) later states: "When stilbestrol was administered hyperglycemia and glycosuria were induced in over two-thirds of a large series of animals studied. Daily doses as small as 50 gamma were effective. Adaptation occurred so that the diabetic state disappeared after being continually present for periods of 2 to 36 days". After the first month of injecting diethylstilbestrol all the surviving animals remained normoglycemic and aglycosuric, a persistent condition of the treated animals even when their controls became diabetic.

The mechanism of the effect of diethylstilbestrol might be: a) a probably direct hypertrophy of the Langerhans islets, because in unpublished experiments we found that hypertrophy is produced by the local administration of estradiol in the pancreas of rats, guinea pigs and cats; b) probable hypofunction with increase in weight of the pituitary and of the adrenal glands, in spite of the fact that Ingle(8) showed that the initial action of diethylstilbestrol took place even in the absence of these two glands; c) a direct effect on the liver; d) a peripheric action.

The protective effect upon diabetes and hyperplasia of the Langerhans islets was observed in normal rats and also in those deprived of pancreas and adrenals. Further details on these last experiments will appear in a future publication.

*Summary*. Castrated female rats, with surgical ablation of 95% of the pancreas and maintained under forced feeding were injected with stilbestrol.

In some of these animals, immediately after stilbestrol administration, a transitory stage lasting for about one month, with an increase of the blood sugar level and glycosuria values was observed, followed by a per-

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7. Ingle, D. J., *Am. J. Physiol.*, 1941, v133, 337.

8. Ingle, D. J., *Endocrinology*, 1944, v34, 361.

manent protective action in all the stilbestrol treated rats with normal values in blood sugar and glycosuria, in contrast with the increase of these values observed in non-injected controls.

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## Some Effects of Protein Depletion and 2-Acetylaminofluorene on Activity of Rat Plasma Pseudocholinesterase.* (17669)

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McCance, Widdowson and Hutchinson(1) demonstrated that starvation resulted in a considerable decline in the pseudocholinesterase activity of human plasma. Mendel, Hawkins and Nichikawara(2) reported similar results in rats. The following experiments were planned to determine whether or not this decrease in pseudocholinesterase activity was associated with depletion in protein stores. The activity of this enzyme was also studied in animals fed 2-acetylaminofluorene, where depletion in protein and other signs of inanition have been noted incident to the development and growth of tumors.

*Methods.* A. *Enzyme Assay.* The Warburg manometric method of pseudocholinesterase determination as described by Ammon(3) and modified by Mendel and Mundell(4) was used to estimate enzyme activities. All determinations were done in duplicate; 3 readings were taken at 20 minute intervals and, after summing, the activities were expressed as $\mu$l $CO_2$/ml plasma/hr. The buffer system was bicarbonate-$CO_2$ at pH 7.4. The substrate was benzoylcholine chloride (Hoffmann-LaRoche) at a final concentration of 0.006 M. Blood was obtained from ether anesthetized animals by heart puncture with a lightly oxalated syringe.

B. *Liver Protein Assay.* Liver protein was determined by applying the factor of 6.25 to micro-Kjeldahl N determination. The liver was removed from the animal immediately upon sacrifice and a sample homogenized for analysis.

C. *Diets and Animals.* Male Wistar rats were used throughout. Control animals were fed a semi-synthetic diet compounded as follows, the values being expressed as percentages. Casein 18, sucrose 13.4, glucose 20.2, dextrin 18.2, lard 24.2, Wesson's salt mixture(5) 1.7, agar 3.3, cod liver oil 1, water added equal to 1.4 times the dry weight. Vitamin supplements, as mg/kg wet weight of diet were, choline chloride, 1000 mg; inositol, 100; riboflavin, 3.2; thiamin, 2; ascorbic acid, 4; pyridoxine HCl, 1.6; 40 mg each of Ca Pantothenate, niacin, p-aminobenzoic acid and $\alpha$-tocopherol and 0.2 each of 2-methyl-1, 4-naphthoquinone biotin and folic acid. Water was added so that the diet might be fed as a gel, thus reducing scattering and allowing a more accurate estimation of uneaten food. For animals to be fed the protein-free diet, glucose was substituted isocalorically for the casein of the control diet. For animals to be fed the "drug" diet, 0.05% 2-acetylaminofluorene was added to the control diet.

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* This work was done in part under a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council. In partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[†] Major, USAF. Studying at Rutgers University under the provisions of Air Force Letter 50-21, dated August 6, 1947.

1. McCance, R. A., Widdowson, E. M., and Hutchinson, A. O., *Nature*, 1948, v161, 56.

2. Mendel, B., Hawkins, R., and Nichikawara, M., *Am. J. Physiol.*, 1949, v154, 495.

3. Ammon, R., *Pflug. Arch. ges. Physiol.*, 1933, v233, 486.

4. Mendel, B., and Mundell, D. B., *Biochem. J.*, 1943, v37, 64.

5. Wesson, L. G., *Science*, 1932, v75, 339.

TABLE I.
Plasma Pseudocholinesterase Activities and Liver Protein Concentrations of 4 Protein-starved Rats Compared with 4 Pair-fed Controls.

| | 42 days protein-free diet | Pair-fed controls |
|---|---|---|
| Pseudocholinesterase activity ($\mu$l $CO_2$/ml/hr) | 14, 41, 34, 23 (28)* | 79, 64, 75, 54 (68)*<br>† t = 9.5; P = <0.01 |
| Liver protein conc. (g protein/100 g liver) | 11.5, 12.4, 11.0, 9.6 (11.1)* | 19.3, 19.0, 19.2, — (19.2)* |

* Means.

† "t" and "P" values are in relation to the pseudocholinesterase values obtained for the two groups.

*Results*. A. *Characterization of Rat Plasma Pseudocholinesterase*. Since rat plasma pseudocholinesterase specifically has not been characterized, it was deemed necessary to establish the identity of the rat and dog enzymes, the latter having been thoroughly studied(6). Accordingly, curves relating enzyme activity to *pH, enzyme concentration,* and *substrate concentration* were obtained, using the pooled plasma of 22 rats. These curves were in good agreement with the data recorded for the dog plasma enzyme by Mendel, Mundell and Rudney(6). Since Warburg determinations to be described were extensive and spread over a period of approximately 14 days, plasma was used in many cases, that had been stored in the frozen state. To test enzyme stability, plasma was pooled from 5 rats and divided into 5 equal portions. Portion 1 was assayed for pseudocholinesterase activity immediately upon being taken from the animal. Portions 2 and 4 were stored at 4°C and portions 3 and 5 were stored frozen, at –19°C. After 7 days, portions 2 and 3 were assayed for peudocholinesterase activity and, after 14 days, portions 4 and 5 were assayed. The values obtained, for the 5 portions respectively were: 119.6, 121.7, 119.0, 118.0, and 131.1. The plasma is therefore very stable (whether stored at 4°C or frozen at –19°C) with respect to enzyme activity.

B. *Protein Depletion*. Eight rats, ages approximately 6 months, weights 200-250 g were divided into 2 groups. The first group was fed the protein-free diet for 6 weeks while the second group was pair-fed on the control diet. At 42 days all rats were sacrificed, their plasmas assayed for pseudocholinesterase activity and their livers for protein concentration. The data in Table I show that the fall in liver protein induced by protein starvation is accompanied by a decrease of plasma pseudocholinesterase activity to approximately 50% of control level. The average protein concentration in the livers of control rats agrees well with data from the literature(7) and with data obtained from similar experiments in this laboratory. Likewise, evidence of severe depletion in the animals fed the protein-free diet is given when the protein concentration of their livers is compared with values reported in similar experiments by Kosterlitz(7). Thus it would seem that a depletion in liver protein is correlated with a fall in the level of rat plasma pseudocholinesterase activity.

C. *2-Acetylaminofluorene*. Eight groups of rats were placed on experiment for a period of 32 weeks. Four groups were maintained throughout on the control diet. The remaining 4 groups received the "drug" diet for a period of 14 weeks and thereafter were given the control diet, free of the drug. At 8, 14, 24 and 32 weeks respectively, a group of the drug-fed animals and a group of the control rats were sacrificed. Plasmas were assayed for pseudocholinesterase activity, liver proteins were determined and the incidence of tumors noted. Table II indicates that after 8 weeks on a diet containing 2-acetylaminofluorene, the level of liver protein in the drug-fed animals did not differ significantly from the corresponding level in pair-fed controls. Plasma pseudocholinesterase activity, on the

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6. Mendel, B., Mundell, D. B., and Rudney, H., *Biochem. J.*, 1943, v37, 473.

7. Kosterlitz, H. W., *J. Physiol.*, 1947, v106, 194.

TABLE II.
Liver Protein, Pseudocholinesterase Activity of the Plasma and Hepatoma Incidence in Rats Fed 2-acetylaminofluorene and in Pair-fed Controls. Four animals in each group—a control group and a drug group sacrificed at the end of each period.

| | Liver protein conc. g protein/100 g liver | | Pseudocholinesterase activity $\mu l\ CO_2/ml/hr$ | | | | Hepatomas, % | |
|---|---|---|---|---|---|---|---|---|
| Wks | Controls | Drug† | Controls | Drug† | *''t'' | *''P'' | Controls | Drug† |
| 8 | 18.7 | 17.5 | 92 | 193 | 7.9 | $<.01$ | 0 | 0 |
| 14 | 16.7 | 17.4 | 71 | 169 | 3.3 | .015 | 0 | 50 |
| 24 | 19.2 | 15.9 | 68 | 160 | 7.4 | $<.01$ | 0 | 75 |
| 32 | 18.7 | 12.4 | 86 | 110 | 2.5 | .04 | 0 | 100 |

* ''t'' and ''P'' values refer to the relationship of the pseudocholinesterase values obtained for the drug-fed animals to those obtained for the control animals for each period.

† Drug withdrawn after 14 wks.

other hand rose to a level approximating twice that of control values. After 24 weeks, at which time the drug-fed animals displayed the initial evidence of decrease in liver protein concentration, pseudocholinesterase activity values were still significantly higher than control values. After 32 weeks, when liver protein concentration was quite low in the animals fed 2-acetylaminofluorene, plasma enzyme activity remained slightly higher than corresponding control levels. It should be noted however, that the total liver protein increased in the drug-fed animals as a result of an increase in size of the liver. This increase in size was associated with abnormally high water and fat concentrations but with an abnormally low protein concentration.

The mechanisms by which plasma pseudocholinesterase is stimulated by the feeding of 2-acetylaminofluorene remains obscure at this time. Attention is drawn, however, to the fact that the rise in plasma pseudocholinesterase activity precedes any gross evidence of hepatoma, and that the activity of the enzyme does decrease significantly following this rise,—a decrease which may be associated with depletion. These observations are receiving further study.

*Summary.* Evidence is presented for the identity of the plasma pseudocholinesterase of dog and rat and that the latter enzyme is stable when stored at low temperatures. Protein starvation is accompanied by a decrease in rat plasma pseudocholinesterase activity. The activity of this enzyme has been shown to increase markedly in rats fed 2-acetylaminofluorene before any gross evidence of hepatoma can be detected. As the hepatoma develops and liver protein concentration declines, the pseudocholinesterase activity of the plasma decreases from this high level.

The author is indebted to Dr. James B. Allison for frequent advice and guidance and also to Dr. Walter W. Wainio for helpful suggestions during the course of this study.

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## Methylene Blue Potentiation of Alloxan Diabetes.* (17670)

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Highet and West(1) reported that the intraperitoneal injection of methylene blue (1 cc of a 1% solution per rat), administered 30 minutes prior to alloxan protected

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* Aided by a grant from the Cleveland Diabetes Fund.

1. Highet, Doris H., and West, Edward S., *J. Biol. Chem.*, 1949, v178, 521.

TABLE I.
The methylene blue was injected intravenously into the tail vein in doses of 35-50 mg/K (3.5-5.0 cc of 1% MB/K) immediately preceding the alloxan (30-40 mg/K).

| No. of animals | Avg initial wt | Alloxan dose, mg/K | Methylene blue dose, mg/K | % diabetic | P value | Avg 48-hr blood sugar | |
|---|---|---|---|---|---|---|---|
| | | | | | | Diabetic | Non-diabetic |
| 6 | 149 | 40 | 0 | 100 | | 389 | — |
| 3 | 156 | 40 | 50 | 100 | | 500 | — |
| 44 | 142 | 30 | 0 | 52 | | 338 | 119 |
| 17 | 136 | 30 | 35 | 100 | < .001 | 420 | — |

rats against diabetes. The alloxan was also injected intraperitoneally, in doses of 150 mg/K. Since we previously found that the production of diabetes by intraperitoneal injection of alloxan was subject to great variation, as compared to the diabetes produced by its intravenous injection, and since this mode of administration required about 5 times the dose of alloxan(2), it was thought desirable to repeat the methylene blue experiments using the intravenous route.

*Methods.* Sprague Dawley rats were used in this study. They were allowed food and water *ad lib.* The alloxan monohydrate solutions were prepared in concentrations of 1.5 and 2.0% and they were injected intravenously (0.2 cc per 100 g of body weight) in doses of 30 and 40 mg/K. The technique of intravenous injection and blood sampling was carried out in the manner previously described(2). Methylene blue was prepared as a 1% aqueous solution and was injected intravenously in doses ranging from 3.5 to 5.0 cc/K (35-50 mg/K). Since the dose of methylene blue used by Highet and West was 1 cc of 1% methylene blue per rat (rats weighing between 189 and 310 g) this dose was equivalent to 32-53 mg/K. Blood samples were drawn for sugar determinations by the Folin Malmros micro method(3) at 24, 48, and 72 hours after the injection of the alloxan. The rats were considered diabetic if any of the blood sugar values were greater than 150 mg %. As a rule the 48 hour blood sugar values were in the neighborhood of 300 to 400 mg %.

*Experimental. Potentiating action of methylene blue on alloxan diabetes.* The effect of methylene blue injection on the production of alloxan diabetes is shown in Table I. It was previously established(2) that when 40 mg/K of alloxan were injected into fed rats, 95% were rendered diabetic. The 6 rats injected with this dose agreed with this. However, only 3 of the 6 rats which received methylene blue intravenously (50 mg/K) immediately before being given this alloxan dose survived and all of these developed diabetes. Their average 48 hour blood sugar value was higher (500 mg%) than was the case in the rats receiving only alloxan (389 mg %).

When the intravenous alloxan dose was reduced to 30 mg/K, diabetes was produced in only 52% of the 44 rats injected. When methylene blue was injected in doses of 35 mg/K immediately preceding this dose of alloxan all of the rats developed diabetes and again the average 48 hour blood sugar value was higher.

These findings disagree with those reported by Highet and West. The intravenous injection of methylene blue immediately preceding the alloxan, sensitized the rats to the development of alloxan diabetes. Both the incidence and severity of the diabetes was increased. Statistical analysis of the data indicates that the potentiating action of methylene blue is highly significant ($P < .001$).

Since we injected the methylene blue immediately preceding the alloxan, whereas Highet and West injected the methylene blue 30 minutes before, it seemed possible that their reported protection might be due to a delayed effect which appeared after a latent period. Eight rats were therefore injected

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2. Lazarow, A., and Palay, S. L., *J. Lab. and Clin. Med.*, 1946, v31, 1004.

3. Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, v83, 115.

with methylene blue (35 mg/K) and after 60 minutes were given a diabetogenic dose of alloxan (40 mg/K i.v.). However, as in the previous experiments, no significant protection was observed.

*Reactions of methylene blue with glutathione and dialuric acid, and the mechanism of methylene blue potentiation.* It has been reported that methylene blue is reduced by glutathione and cysteine(4). Since glutathione injection protects rats against the development of alloxan diabetes(5,6), any factor which lowers the concentration of reduced glutathione should sensitize animals to the development of alloxan diabetes(7). However, in preliminary studies carried out *in vitro* under anaerobic conditions at pH 7.4, glutathione, in concentrations of 600 mg/L, (which is equivalent to a blood GSH of 60 mg/100 cc) was found to reduce methylene blue (35 mg/L) only slightly in 30 minutes.

When alloxan is injected into an animal it is destroyed by several reactions, one of which is its reduction to dialuric acid. This can be accomplished by cysteine(8,9,10), glutathione(9,10), and the reduced coenzyme DPN-$H_2$(11). It now seems fairly well established that when alloxan is reduced to dialuric acid it loses its diabetogenic potency(9). Cysteine, which rapidly accomplishes this reduction *in vitro*(8,12), also protects rats against alloxan diabetes(6). It would also be expected that any compound which is capable of reoxidizing the dialuric acid thus formed back to alloxan, might potentiate the production of diabetes. We have found in preliminary studies that visible reduction of methylene blue occurs within 5 minutes when dialuric acid (40 mg/L) was added to a methylene blue solution (35 mg/L) anaerobically at pH 7.4. It may be presumed that the methylene blue oxidizes the dialuric acid to alloxan.

*Discussion.* Although we are unable to account for the reported findings of Highet and West, the following may possibly explain their results: (1) Diabetes may have been produced, but it may have been missed because of a delayed hypoglycemic or norglycemic phase which occasionally occurs as late as 48 or 72 hours(2). (2) The intraperitoneal injection of near toxic doses of methylene blue modified the physiological state of the peritoneal cavity and interfered with the subsequent absorption of alloxan. Since alloxan is rapidly decomposed to a non-diabetogenic compound at pH 7.4(9,12), such a delay in its absorption would be equivalent to decreasing the injected dose of alloxan.

The possibility that alloxan or dialuric acid may occur within the human body has been considered(7). The potentiating action of methylene blue serves to emphasize that potential diabetogenic compounds which might normally be present within the human body could be converted to active diabetogenic compounds under appropriate conditions. Recently Patterson has shown that large doses of the oxidized form of vitamin C, *i.e.* dehydroascorbic acid will produce a permanent diabetes in rats(13). Incidentally, both dialuric acid(11) and ascorbic acid(14) are oxidized to their respective diabetogenic compounds by cytochrome *c*. Therefore the possibility that abnormal metabolic conditions may favor the appearances of diabetogenic compounds within the human body needs careful investigation.

*Summary.* 1. Methylene blue injected intravenously in doses of 35 mg/K immediately preceding alloxan (30 mg/K i.v.) increased

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4. Black, Maurice M., *Cancer Res.*, 1947, v7, 592.

5. Lazarow, A., *Anat. Rec.*, 1945, v91, 24.

6. Lazarow, A., PROC. SOC. EXP. BIOL. AND MED., 1946, v61, 441.

7. Lazarow, A., *Phys. Rev.*, 1949, v29, 48.

8. Archibald, R. M., *J. Biol. Chem.*, 1945, v158, 347.

9. Lazarow, A., Patterson, J. W., and Levey, S., *Science*, 1948, v108, 308.

10. Patterson, J. W., Lazarow, A., and Levey, S., *J. Biol. Chem.*, 1949, v177, 197.

11. Cooperstein, S. J., Lazarow, A., and Patterson, J. W., to be published.

12. Patterson, J. W., Lazarow, A., and Levey, S., *J. Biol. Chem.*, 1949, v177, 187.

13. Patterson, J. W., *J. Biol. Chem.*, 1950, in press.

14. Szent Gyorgyi, A., *Biochem. J.*, 1928, v22, 1387.

the incidence of diabetes from 52 to 100%.

2. The potentiating effect of methylene blue is attributed, in part at least, to its ability to reoxidize dialuric acid back to alloxan and thereby in effect it is equivalent to increasing the effective dose of alloxan.

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# Absorption, Excretion, and Toxicity of Milibis* (Bismuthoxy-p-N-glycolylarsanilate) Following Oral Administration. (17671)

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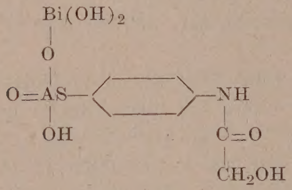
Milibis, a drug which promises to become important in the treatment of amebiasis, is a salt-like complex derived from bismuth trioxide and p-N-glycolyarsanilic acid. It has the approximate composition $C_8H_9AsBiNO_6 \cdot xH_2O$, and was first studied by Hauer(1) under the name of "Wia". The probable structure of its principal component, bismuthoxy-p-N-glycolylarsanilate, is as follows:



In the product as supplied, a small amount of the di-substituted compound $(C_8H_9AsNO_5)_2$-BiOH, is probably also present. Milibis has been found quite effective as an amebicide in both experimental animals(2,3) and human subjects(1,2,4-6) as well as for the treatment of experimental yaws and syphilis in rabbits(2). From the very low aqueous solubility of the product† it could be predicted that only small amounts would be absorbed following oral administration, but obviously the product must dissolve to some extent if microorganisms are to be attacked. In view of the toxic potentialities of As and Bi compounds, it has been of interest to determine specifically the amounts of these elements absorbed following oral administration of the drug, and to ascertain whether appreciable quantities tend to accumulate in the tissues.

*Acute toxicity*. No toxic manifestations were apparent following single oral doses of 2.5, 5, or 10 g/kg, in either mice or rats. Groups of 3 rats, and 9 mice, were used at each dose level.

*Subacute toxicity*. Four groups of 5 rats weighing initially 130-140 g were given single oral doses of 2.5, 5, 7.5, and 10 g/kg by stomach tube, on 6 days of each week for 3 weeks. The drug was administered as a suspension in 0.5% aqueous gum tragacanth, and in a volume of 2 cc/100 g. Food (Purina Dog Checkers) and water were provided *ad lib.* The rats were weighed frequently so that comparisons could be made with a control group of 5 animals. One rat in the 5 g/kg and 2 rats in the 10 g/kg group did not survive the experiment. Two of these deaths were obviously accidental, due to difficulties in administering the heavy suspensions of the drug and only the one death at the highest dose level could be attributed to the drug. The average % weight gains of the surviving animals during the 21 days were as follows:

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* Registered trademark of Winthrop-Stearns Inc.

1. Hauer, A., *Deutsche Trop. Z.*, 1943, v47, 153; *O. P. B. Report* No. 237, 1945, p. 38.

2. *O. P. B. Report* No. 981, 1945, p. 28.

3. Dennis, E. W., Berberian, D. A., and Hansen, S., *Am. J. Trop. Med.*, 1949, v29, 683.

4. Berberian, D. A., *J. Clin. Invest.*, 1948, v27, 525.

5. Garza Brito, A. de la, and Treviño Villaseñor, A., *Rev. Med. Mex.*, 1949, v29, 261.

6. Dennis, E. W., Berberian, D. A., and Tainter, M. L., *Prensa Med. Mex.*, 1949, v14, 115.

† The solubility of Milibis in water, ethanol, acetone, and propylene glycol is less than 0.025% at room temperature.

Some specific solubilities (per 100 cc of solvent) are as follows: 20% ethanol, 0.2 mg; 30, 50, and 95% ethanol, each 0.3 mg; 0.1 N HCl, 1.5 mg. These figures were obtained by determining the Bi content of a solution which had been saturated at room temperature, and calculating the Milibis content therefrom.

| | |
|---|---|
| Control group | 51 |
| 2.5 g/kg/day group | 48 |
| 5.0 ,, ,, | 51 |
| 7.5 ,, ,, | 43 |
| 10.0 ,, ,, | 33 |

Thus only at the highest dose level was the growth of the animals significantly inhibited, and this may simply have been the result of the administration of such a large bulk of indigestible material. At autopsy no gross or microscopic pathological changes attributable to the drug were found in any of the animals.[‡] It may be concluded, therefore, that rats are completely tolerant to oral doses of 5 g/kg/day for 3 weeks, under the conditions of the experiment.

*Methods for metabolic studies.* Male hooded rats weighing 240-270 g were kept in metabolism cages, one pair to each cage, with food[§] and water being provided *ad lib*. The drug was incorporated in the diet at a level of 0.15%. It was estimated that the daily food consumption of the rats would be about 20 g, hence the expected intake of drug was slightly in excess of 100 mg/kg/day. The animals remained on the drug-containing diet for 12 days, with an accurate record of their food consumption being kept. For the next 24 hours they received the same diet without drug, then they were killed, frozen, and stored for subsequent analysis. Excreta were collected during the 13-day period, with the feces being separated from the urine on wire screens. For analytical purposes the excreta were combined for the first 6, and for the last 7 days of the experiment. The urine samples were centrifuged; the precipitated solids were added to the feces, which were then thoroughly dried, ground, and mixed before sampling. An aliquot of the clear supernatant urine was taken for analysis.

Methods used for the determination of As and Bi in food, urine, and tissues have been described elsewhere(7,8). A sample of the drug-containing diet was found on analysis to contain 0.557 mg Bi and 0.212 mg As per g. Since the particular lot of drug used in this work contained 36.8% Bi and 14.3% As,[‖] the calculated amounts for 0.15% Milibis in diet are 0.552 mg Bi and 0.215 mg As per g. The procedure used for the digestion of feces represents a slight modification of those previously described. It gave the most consistent and most nearly quantitative recoveries of As and Bi:

Transfer 1.00 g dry, finely powdered feces and a few glass beads to a 300 cc Kjeldahl flask. Add 25 cc conc. C.P. nitric acid and boil very gently for about 20 min. Cool briefly, add 17 cc conc. C.P. sulfuric acid, then boil more actively. Add further small quantities of nitric acid as needed so that an excess is always present. Finally remove the last traces of color in the digest with a few drops of perchloric acid. Cool, add 30 cc water and boil it off; repeat the boiling-off process. Cool the digest and dilute it accurately with water to 100 cc. It is suitable for the determination of both As and Bi by the methods specified. When known amounts of Milibis were digested in the presence of 1 g dry rat feces the recoveries were as follows: As, 96%; Bi, 97.5%.

For analysis of representative tissues of the animals, the livers, kidneys, and spleens were removed, the tissues of each animal of a pair being treated separately. Digests were made of 2 to 3 g liver, of both kidneys, and of the whole spleen. At the same time the gastrointestinal tracts were dissected out, and the entire contents were removed for analysis. The results of this experiment are presented in Table I.

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[‡] Pathological examinations were made by Dr. F. C. Goble.

[§] Percentage composition: Ground yellow corn and ground whole wheat, each 28.5; whole milk powder, 23.0; linseed meal, 8.0; commercial casein, 4.1; alfalfa and brewer's yeast, each 3.0; cod liver oil, 0.9; calcium carbonate and sodium chloride, each 0.5.

7. McChesney, E. W., *Anal. Chem.*, 1949, v21, 880.

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[‖] Data supplied through the courtesy of Mr. M. E. Auerbach.

TABLE I.
Absorption and Excretion of Arsenic and Bismuth in Rats Receiving Milibis in the Diet at About the Level of 100 mg/kg/day for 12 Days.

| | | Drug intake | | Arsenic metabolism* | | | | | Bismuth metabolism* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pair | Interval, days | Total, mg† | mg/kg/day‡ avg | Intake§ | Urinary output | Fecal output | Digestive tract¶ | Total | Intake‖ | Urinary output | Fecal output | Digestive tract¶ | Total |
| 1 | 1- 6 | 333 | 105 | 48.0 | 1.7 | 37.2 | — | 38.9 | 123.0 | 0.4 | 102.3 | — | 102.7 |
| | 7-13 | 340 | 117 | 48.8 | 2.2 | 46.4 | — | 48.6 | 125.4 | 0.4 | 132.5 | — | 132.9 |
| | 1-13 | 673 | 111 | 96.8 | 3.9 | 83.6 | 0.1 | 87.7** | 248.4 | 0.8 | 234.8 | 0.3 | 235.9 |
| 2 | 1- 6 | 338 | 104 | 48.4 | 1.2 | 35.8 | — | 37.0 | 124.0 | 0.4 | 98.6 | — | 99.0 |
| | 7-13 | 403 | 134 | 57.7 | 1.6 | 55.6 | — | 57.2 | 148.1 | 0.5 | 160.0 | — | 160.5 |
| | 1-13 | 741 | 119 | 106.1 | 2.8 | 91.4 | 0.2 | 94.5** | 272.1 | 0.9 | 258.6 | 0.5 | 260.0 |
| 3 | 1- 6 | 350 | 111 | 50.0 | 1.5 | 39.6 | — | 41.1 | 128.5 | 0.5 | 108.0 | — | 108.5 |
| | 7-13 | 357 | 123 | 51.2 | 1.7 | 47.0 | — | 48.7 | 131.5 | 0.5 | 132.0 | — | 132.5 |
| | 1-13 | 707 | 117 | 101.2 | 3.2 | 86.6 | 0.2 | 90.1** | 260.0 | 1.0 | 240.0 | 0.5 | 241.5 |
| 4 | 1- 6 | 232 | 80 | 33.6 | 0.9 | 25.3 | — | 26.2 | 86.0 | 0.3 | 71.0 | — | 71.3 |
| | 7-13 | 315 | 117 | 45.2 | 1.5 | 42.5 | — | 44.0 | 116.0 | 0.7 | 116.0 | — | 116.7 |
| | 1-13 | 547 | 98.5 | 78.8 | 2.4 | 67.8 | 0.4 | 70.7** | 202.0 | 1.0 | 187.0 | 0.7 | 188.7 |
| 5 | 1- 6 | 345 | 112 | 49.5 | 2.1 | 39.5 | — | 41.6 | 126.8 | 0.6 | 106.5 | — | 107.0 |
| | 7-13 | 359 | 122 | 51.4 | 1.9 | 50.4 | — | 52.3 | 132.0 | 0.6 | 143.0 | — | 143.6 |
| | 1-13 | 704 | 117 | 100.9 | 4.0 | 89.9 | 0.1 | 94.0** | 258.8 | 1.2 | 249.5 | 0.5 | 251.2 |

* All figures given under these headings are in mg.
† 0.15% of net food consumption for the period.
‡ Drug intake in g, divided by 6 and by avg wt of the 2 rats during the period, in kg.
§ Drug intake × 0.1433.
‖ Drug intake × 0.368.
¶ Analysis of entire contents at autopsy on 13th day (stomach, intestines, and cecum).
** Includes 0.1 mg in liver, kidney, and spleen.

TABLE II.
Urinary Excretion of Arsenic* (as mg) by Human Subjects Taking Doses of 0.5 g Milibis 3 times daily for 10 Days.†

| Day of exp. | Subjects | | | | |
|---|---|---|---|---|---|
| | A.A. | D.B. | C.B. | E.F. | G.W. |
| 1 | 0.5 | 1.1 | 2.9 | 1.6 | 0.5 |
| 4 | 2.6 | 1.3 | 1.9 | 2.6 | 0 |
| 8 | 2.2 | 2.5 | 3.2 | 3.1 | 0 |
| 10 | 3.4 | 1.4 | 1.4 | 2.3 | 0 |
| 11 | 1.3 | 0.2 | 0.4 | 1.2 | 0 |
| 12 | 0.9 | 0 | 0 | 1.4 | 0 |

* No bismuth was found at any time. The urine of all subjects was demonstrated to be arsenic and bismuth-free on a sample collected during the 24 hr before the first dose.

† The daily dose contained 215 mg As, 552 mg Bi.

*Excretion of arsenic and bismuth by human subjects following the oral administration of Milibis.* Five adult males weighing 62-84 kg took 2 tablets of 0.25 g each 3 times daily (total of 1.5 g) for 10 days. This is the recommended therapeutic course of Milibis, and amounts to about 20 mg/kg/day, or slightly less than 20% of the daily dose received by the rats. Twenty-four-hour urine samples were collected for Days 1, 4, 8, 10, 11, and 12 following the first dose. The urine samples were analyzed for As and Bi as described above. No Bi was detected in the urine at any time, which means that less than 0.2 mg was being excreted through the kidneys daily. The amounts of As excreted are presented in Table II; it will be noted that in 4 subjects the output was from 1 to 3 mg/day while the fifth subject excreted almost none. Two of the subjects were still excreting small amounts or arsenic 2 days after the last dose. Routine and microscopic analyses of the urine samples were conducted¶ with entirely negative results. The only untoward effect resulting from the medication was an occasional extra bowel movement.

*Discussion: Arsenic metabolism.* From 3 to 4% of the ingested As was excreted in the urine of the rats. Although urine samples were not collected from the human subjects for all of the entire experimental period, it is evident that they excreted a somewhat smaller proportion. In rats the fecal output accounted for almost all of the drug. A very small amount remained in the gastrointestinal tract 24 hours after the drug was removed from the diet. Analysis of the tissues revealed that the amounts of As present were so small as to be almost undetectable by the method used. For example, the whole liver of a single animal contained 0.04-0.08 mg, the spleen contained about 0.01 mg, and the 2 kidneys, 0.006-0.008 mg.

The recovery of arsenic as against the amount ingested, however, was not quantitative. Even after making allowance for the fact that only 96% of As added to feces as Milibis could be recovered by the analytical method used, it was still not possible to account for about 5% of the amount ingested. However, losses of greater magnitude have been observed in As balance studies conducted by others(9). In view of the known affinity of tissues for As following the administration of arsenicals(10), it was considered unlikely that significant amounts would be found in sites other than those mentioned above. Nevertheless, in order to establish this point more positively, the remainders of the carcasses of pair 3 were brought completely into solution in hot concentrated nitric acid, and small aliquots of these solutions were further digested as described for feces. It was found that an aliquot representing 1/400 of a carcass contained no detectable Bi (*i.e.* less than 0.005 mg), while an aliquot representing 1/40 of a carcass contained 0.007-0.008 mg As. If all the ingested As and Bi which was not accounted for had been contained in the carcasses, the amounts found in these aliquots (assuming equal distribution between the 2 animals) would have been 0.023 mg Bi and 0.137 mg As.

Other particular sites of localization which are worthy of some consideration are muscle and blood. We have previously(11) carried out experiments identical in every respect with those reported here except that the medications were given daily by stomach tube, suspended in aqueous gum tragacanth. The rats were killed 24 hours after receiving the eleventh consecutive dose of either 100 or 200 mg/kg/day. There was no detectable As in the muscles of these animals, and only 0.3 mg in the whole blood volume of a pair. The amount of 0.3 mg applied very closely to all pairs of animals on the test, and is almost the least detectable by the method used. Also, by way of confirmation of the present work, the entire livers of 2 animals contained 0.04 - 0.11 mg As, the 2 spleens contained 0-0.46 mg, and the kidneys, none.

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¶ These observations were made by Dr. D. A. Berberian.

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10. Raulston, B. O., and Magnuson, H. J., *Trans. Assn. Am. Physicians*, 1940, v55, 254.

11. McChesney, E. W., unpublished observations.

*Bismuth metabolism.* Practically no Bi was absorbed as a result of ingesting Milibis. The average urinary output of the rats was only 0.4% of the intake, and in human subjects the amount (if any) was too small to be measured. Fecal excretion accounted for almost the entire intake of rats; making due allowance for the amounts of bismuth as Milibis known to be recoverable from feces, about 2% of the intake was not accounted for in most of the pairs. In the previously conducted experiments(11) the kidneys from a pair of animals contained only a trace of bismuth; *i.e.,* 0.1-0.5 mg, while the livers, spleens, muscles and blood contained none. These are the tissues which are most likely to contain Bi(12), and its absence from the remainders of the carcasses has been demonstrated (see above).

The data on both As and Bi metabolism support the idea that bismuthoxy-mono-p-N-glycolylarsanilate remains largely unabsorbed in its passage through the gastrointestinal tract. It is nevertheless clearly necessary that some solution, however slight, should occur if there is to be any amebicidal action. It is also evident that solution does occur. In this connection there are two apparent possibilities: First, the small amount of $As^{5+}$ which is absorbed may originate in the bismuthoxy-di-p-N-glycolylarsanilate which is evidently present in the complex, since the second arsanilate radical would be more loosely held. In this sense the bulk of the drug would act as a carrier for the small amount of the amebicidally active component. Second, the compound may undergo slight hydrolysis in the stomach, releasing $Bi^{3+}$ (which then becomes insoluble $Bi_2O_3$ in the gut) and the soluble arsanilic acid, which acts against the microorganisms. As a result of hydrolytic action, for example, Milibis seems to have a slight solubility in 0.1 N HCl,† while in N HCl it is very soluble.

In any case, the amount of As absorbed following the oral administration of Milibis is much less than that observed following the administration of Carbarsone(13) or of the thioarsenites C.C. 937 and C.C. 1014(14), compounds which have also been reported to be effective for the treatment of amebiasis. The method by which Milibis has been administered to the rats in this work; namely, the almost constant introduction of small amounts, should result in maximal absorption. The relatively minor amounts of As and Bi which were absorbed, at a level 5 times the human therapeutic dose, are in harmony with the demonstrated low laboratory and clinical toxicity of the drug.

*Summary.* Milibis is almost completely non-toxic when administered orally to rats. A dose of 10 g/kg/day for 18 of 21 days inhibited growth only slightly, and at this dose level the mortality was 20%; with smaller doses there was no inhibition of growth, and no mortality attributable to the drug. Absorption was studied in human subjects and rats, during and following repeated oral administration. Both species excreted in the urine at most 2 to 4% of the ingested As. Human subjects excreted no detectable Bi in the urine, but the rats excreted about 1% of the amount ingested. In the rats tissue concentrations of both As and Bi were negligible, as was the As concentration in blood.

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# Survival Time of the Mouse as Related to Ambient $pO_2$ Produced by Explosive Decompression. (17672)

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Gelfan, Nims and Livingston(1) have shown that altitudes above 52,500 feet (77 mm Hg do not further reduce survival time of the rat; therefore the critcal $pO_2$ of the ambient air for the rat would be 16 mm Hg. Rats decompressed in oxygen likewise showed no further effects of altitudes above 52,000 feet. However, rats survive more than twice as long when explosively decompressed in oxygen as in air but even then the critical decompression pressure remains the same for both air and oxygen. Luft, Clamann and Adler(2) state that mice survive 25 seconds when rapidly decompressed to 43,000 feet in air or to 52,000 feet in oxygen. They also list survival of mice for 80 seconds at 32,000 fect in air. Hailman(3) using rats, and Stullken and Hiestand(4) using mice and chicks have determined the lowest oxygen pressures compatible with life. The former(3) found the critical $pO_2$ in rats decompressed in oxygen between 65 and 75 mm Hg, the latter(4) found the critical $pO_2$ in mice as 70.7 mm in baby chicks as 89.5 mm. Compared to decompression in oxygen, mice decompressed in air died at an average pressure of 218.3 mm or a $pO_2$ of 45.7 mm.

*Method.* A decompression chamber consisting of a Pyrex flask was attached to a large ballast chamber which in turn was evacuated by a vacuum pump. By opening a connection between the ballast chamber and the decompression chamber the air in the latter was reduced in a fraction of a second to any desired pressure. Survival of the animal was measured with a stopwatch to the nearest second. Sixty-five male and 45 female adult mice were decompressed in this study and the end-point taken as the last visible respiratory movement. Since the level of the mercury in the pressure gauge was adjusted to the barometric pressure at the time of the experiment, no correction factor for standard pressure was necessary. The $pO_2$ was measured by multiplying the ambient pressure by 0.2096. We made no attempt to measure or estimate the alveolar $pO_2$ in the mice due to the fact that breathing became so rapid that an estimation would be invalid.

*Results.* For the sake of simplicity a table of the individual animals is omitted; instead the individual survival time in seconds is plotted against ambient oxygen pressures in Fig. 1. From the distribution of the points on the graph it can readily be seen that the critical $pO_2$ below which survival is unaffected occurs at approximately 21 mm Hg (equivalent to 47,000 feet altitude). At $pO_2$ pressures above this level survival time is longer, the curve becoming a horizontal line at about $pO_2$ of 48 mm. The "curve" resembles that for the rat as shown elsewhere(1). There is a species difference, however, in favor of the rat amounting to an altitude difference of about 5,500 feet, probably due to a species difference in metabolic rate. The "point of diminishing returns" is about $pO_2$ of 48 mm above which pressure survival is no longer related to the ambient oxygen pressure. Britton and Kline(5) using nearly 2000 rats have shown a sex difference in survival, the female surviving longer than the male. This was particularly true at low temperatures. Our findings with mice do not indicate such a difference.

*Summary.* One hundred and ten mice were explosively decompressed to different ambient oxygen pressures and their survival time de-

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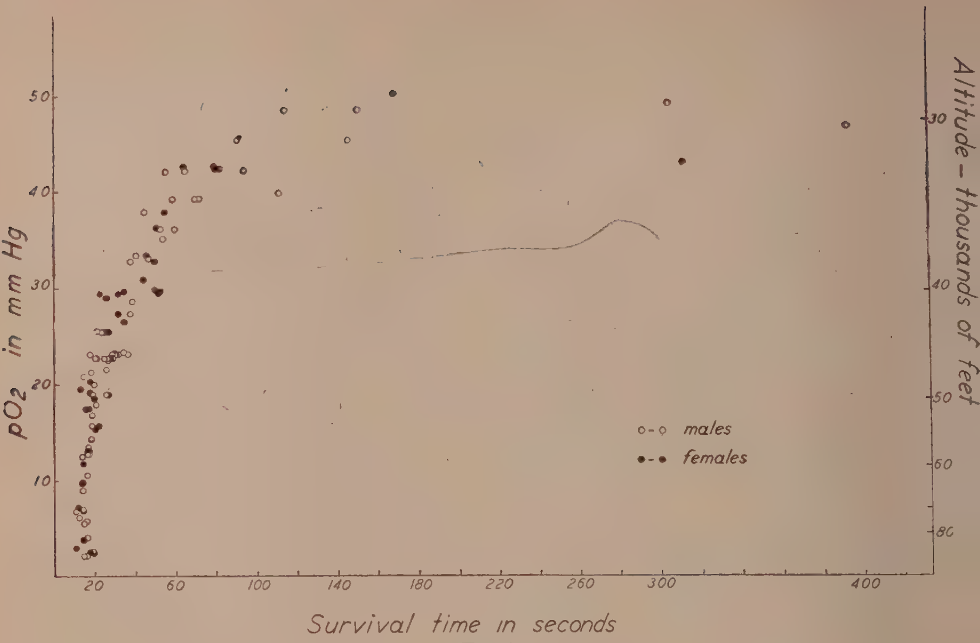

FIG. 1.

termined. A critical barometric pressure of 100 mm Hg exists below which no further reduction in survival time occurs. The $pO_2$ at which survival no longer is dependent on oxygen pressure is therefore about 21 mm Hg. No sex difference in survival of mice was found.

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## Serum Lipids and Their Fractionation in Alloxan Diabetes in the Rabbit.*
(17673)

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In 1945 Kendall *et al.*(1) showed that a transient hyperlipaemia and hypercholesterolaemia may or may not occur during the early stages of uncontrolled alloxan diabetes in the rabbit. Duff and Wilson(2) subsequently confirmed these findings, making a more careful study of the serum cholesterol over a longer period of time. So far as we are aware, however, there has been no detailed study of the other serum lipids in alloxan diabetic rabbits. The lipids of the serum are characterized by two important features: in the first place the different lipid components are known to show an interrelationship which is more constantly maintained and, therefore, of more significance, than the concentration of any single component(3); secondly, but of no less importance, there is a close association between the serum lipids and the serum proteins, and some sort of lipoprotein complex has been suggested to explain the lack of turbidity of normal serum(4). In any detailed analysis of the serum lipids, therefore, it is necessary not only to determine the absolute levels of the various lipid constituents, but also to examine the relationship of these constituents to the serum proteins. Absolute lipid levels can be determined with a reasonable degree of accuracy, but the study of the relationship of the lipids to the serum proteins is fraught with difficulty. The recent publication of Forbes *et al.*(5), however, offered a reproducible method which was considered likely to reflect the state of binding of the lipids in one or more of the serum lipoproteins. The normal range of the serum lipids was first studied in order to establish a base line with which the values obtained from the diabetic sera might be compared. This was of particular importance in regard to the values obtained by the method of Forbes, since serum total cholesterol is the only lipid constituent that has been studied by this method.

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* This work was assisted by a grant-in-aid from the National Research Council, Canada.

† Medical Research Fellow, National Research Council, Canada.

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*Experimental procedure.* Young adult male albino rabbits were used. As a preliminary step forty-two normal animals were studied by the methods described below. For the experiment proper a number of these rabbits were made diabetic by the intravenous administration of Alloxan (Eastman Kodak) in 5% aqueous solution. A single dose of 200 mg per kg of body weight was used. All the animals received the same diet—an unlimited amount of Purina Laboratory Chow and water. Fourteen rabbits became diabetic and maintained a blood sugar of about 300 mg % or more; two rabbits showed only a transitory hyperglycemia and were termed "alloxan recovered". Serum lipid determinations on these sixteen animals were made one or two weeks after the injection of alloxan and subsequently at intervals of about two weeks.

Blood was drawn in the non-fasting state from the central artery of the ear and the serum separated. The presence or absence of visible lipaemia was noted. Half of the serum was used immediately for the determination of the lipid constituents. Total fatty acids were determined by the method of Stoddard and Drury as modified by Man and Gildea(6); lipid phosphorus by the method of Youngburg as modified by Hawk, Oser and Summerson(7); free and total cholesterol by the method of Schoenheimer and Sperry as modified by Sperry(8). Neutral fat was calculated as described by Peters and Man(9). The other half of the serum was rapidly frozen and then dried *in vacuo* and extracted overnight with chloroform in the cold according to the method of Forbes(5). The chloroform was then evaporated *in vacuo* at a temperature below 40°C and determinations of total fatty acids, lipid phosphorus and free and total cholesterol were made on the residue as above. The values obtained in this latter way will be referred to as the "readily extractable lipid fractions", representing, according to Forbes(5), the lipid fractions either not bound or only loosely bound to protein. Blood sugar content was determined by a modification of Folin's micro method(10) at the time of each lipid analysis.

*Results.* Table I summarizes the results of this study. From a consideration of the

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10. *Notes on Operation of Evelyn Photoelectric Colorimeter,* Rubicon Co., Philadelphia, Pa.

TABLE I.
Serum Lipids and Their Fractionation in Alloxan Diabetic Rabbits.

| Date | Wt (kg) | Blood sugar (mg%) | Fatty acids of neutral fat (meq.) | | Lipid phosphorus (mg%) | | Cholesterol (mg%) | | | | | | Visible Lipaemia |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Abs. | | | R.E. | | | |
| | | | Abs. | R.E. | Abs. | R.E. | Total | Free | Ester | Total | Free | Ester | |
| Rabbit A 66 Diabetic—representative of Group I (5 animals). | | | | | | | | | | | | | |
| Nov. 29/48 | 2.86 | 125 | 7.6 | 7.3 | 3.1 | 0.2 | 38 | 13 | 25 | 11 | 6 | 5 | 0 |
| Dec. 2 | 3.18 | Alloxan administered (200 mg/kg) | | | | | | | | | | | |
| ” 15 | 3.35 | 400 | 10.0 | 5.8 | 3.9 | 0.2 | 30 | 20 | 10 | 16 | 3 | 13 | 0 |
| ” 28 | 3.39 | 440 | 7.4 | 5.3 | 3.6 | 0.3 | 46 | 25 | 21 | 34 | 8 | 26 | 0 |
| Rabbit A 61 Diabetic—representative of Group II (8 animals). | | | | | | | | | | | | | |
| Nov. 24/48 | 2.72 | 100 | 5.1 | 5.7 | 2.6 | 0.3 | 50 | 15 | 35 | 14 | 3 | 11 | 0 |
| ” 30 | 2.89 | Alloxan administered (200 mg/kg) | | | | | | | | | | | |
| Dec. 15 | 2.53 | 330 | 88.6 | 65.7 | 20.7 | 14.0 | 270 | 197 | 73 | 208 | 162 | 46 | + |
| ” 28 | 2.49 | 440 | 16.0 | 7.7 | 5.3 | 0.8 | 70 | 30 | 40 | 26 | 7 | 19 | ± |
| Jan. 10/49 | 2.63 | 360 | 9.5 | 3.3 | 3.3 | 0.5 | 55 | 13 | 42 | 30 | 13 | 17 | 0 |
| Rabbit A 59 Diabetic—representative of Group III (2 animals). | | | | | | | | | | | | | |
| Nov. 22/48 | 2.79 | 115 | 6.0 | 3.7 | 4.0 | 0.2 | 22 | 15 | 7 | 10 | 3 | 7 | 0 |
| ” 30 | 2.86 | Alloxan administered (200 mg/kg) | | | | | | | | | | | |
| Dec. 12 | 2.83 | 510 | 226.2 | 202.0 | 46.0 | 36.4 | 615 | 485 | 131 | 470 | 395 | 75 | + |
| ” 28 | 2.83 | 485 | 49.0 | 35.6 | 12.9 | 6.2 | 120 | 83 | 37 | 82 | 50 | 32 | + |
| Jan. 10/49 | 2.83 | 435 | 145.9 | 137.6 | 30.0 | 18.8 | 330 | 265 | 65 | 316 | 257 | 59 | + |
| ” 20 | 2.70 | 520 | 47.0 | 23.0 | 12.5 | 2.7 | 136 | 106 | 30 | 110 | 48 | 62 | + |
| Feb. 1 | 2.98 | 400 | 176.8 | 171.0 | 34.4 | 19.1 | 282 | 113 | 169 | 244 | 130 | 114 | + |
| Rabbit A 4—representative of “Alloxan Recovered” Group (2 animals). | | | | | | | | | | | | | |
| Oct. 31/48 | 3.17 | 95 | 8.2 | 7.4 | 2.8 | 0.2 | 40 | 13 | 27 | 12 | 11 | 1 | 0 |
| Nov. 13 | 3.29 | Alloxan administered (200 mg/kg) | | | | | | | | | | | |
| Dec. 1 | 3.78 | 190 | 6.9 | 2.0 | 2.8 | 0.4 | 18 | 11 | 9 | 16 | 5 | 11 | 0 |
| ” 12 | 4.09 | 120 | 2.9 | 3.2 | 2.7 | 0.3 | 38 | 17 | 21 | 16 | 3 | 13 | 0 |

Note: Abs. = Absolute levels. R.E. = “Readily extractable.”

TABLE II.
Normal Range of Serum Lipids as Determind on 42 Normal Rabbits.

| | Fatty acids of neutral fat (meq.) | | Lipid phosphorus (mg%) | | Cholesterol (mg%) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Total | | Free | |
| | Mean | S.Dev. | Mean | S.Dev. | Mean | S.Dev. | Mean | S.Dev. |
| Absolute lipid levels | 7.3 | 3.1 | 4.4 | 1.7 | 46 | 17.5 | 19 | 9.5 |
| "Readily extractable" lipid levels | 6.2 | 2.7 | 0.3 | 0.01 | 21 | 12.2 | 8 | 6.8 |

absolute lipid levels alone the diabetic animals were found to fall into three groups and for each group the values for one representative animal are given. The determinations in one of the "alloxan recovered" animals are also shown. It will be seen that in the first group, consisting of five animals, there was no conspicuous change in serum lipids; the second group, which consisted of seven animals, showed a brief and temporary rise in serum lipids with a return to normal within three to six weeks; the third group, composed of two animals, showed an elevation in serum lipids that persisted with fluctuations for an indefinite length of time. The two "alloxan recovered" animals behaved like the diabetic rabbits of the first group in that they showed no lipaemia. It was noted that the diabetic animals of group I as well as the "alloxan recovered" animals all gained weight after the administration of alloxan while the diabetic rabbits of group II and III showed a definite loss of weight after the inception of the diabetic state. The rabbits continued to eat well after becoming diabetic and, indeed, the diabetic animals of group III ate ravenously. The diabetic rabbits of group III showed a greater elevation of blood sugar than did those of the other two groups.

Closer study of the absolute lipid levels revealed that when any one of the lipid constituents was elevated, all the others were also elevated, though not to the same degree, and there usually was a visible lipaemia. Relative to normal proportions the fatty acids of neutral fat were most elevated, and the lipid phosphorus least increased; in proportion to the other two lipid constituents, the increase in total cholesterol was intermediate in position. The elevation of total cholesterol was due much more to an increase in free cholesterol than to an increase in ester cholesterol and there was a correspondingly marked change in the ratio between the two. This change in ratio occurred even when there was no rise in the total cholesterol or in the other lipid constituents.

When the "readily extractable" lipid fractions were compared with those obtained by the ordinary methods, several interesting points became evident. Table II shows this comparison in the normal rabbit. It will be seen that in the normal animals the most striking feature was that only a very small portion of the lipid phosphorus was "readily extractable". In addition, the values for total and free cholesterol were usually not more than half of those obtained by routine methods, but varied somewhat from animal to animal. The greater part, if not all, of the fatty acids of the neutral fat were "readily extractable". In the diabetic rabbits when there was a lipaemia, there were definite alterations in this normal ratio between the absolute values and the values for the "readily extractable" lipid fractions. These changes (Table I, Rabbits A61 and A59) were as follows: when the lipid phosphorus was markedly elevated, there was a marked increase in the "readily extractable" fraction of this lipid constituent; the proportion of "readily extractable" total and free cholesterol was also increased when the serum cholesterol was elevated. The proportion of "readily extractable" neutral fat did not change. When there was no lipaemia (Table I, Rabbits A66 and A4) no definite alterations in the "readily extractable" lipid fractions were noted.

*Discussion.* From our results it is clear that alloxan diabetes in the rabbit may or may not be accompanied by an elevation in serum lipids. Whenever an elevation occurred, it was usually transitory, disappearing within six weeks after the inception of the diabetic state, although in a few cases it persisted with fluctuations for an indefinite period of time. In such hyperlipaemia, whether transitory or not, all of the lipid constituents of the serum were increased, the neutral fat showing the greatest increment, a finding which also occurs in the diabetic hyperlipaemia of man(11) and of depancreatized dogs fed raw pancreas before and after insulin deprivation(12). In the diabetic rabbits in which the serum lipids were elevated, there was always a concomitant loss of body weight and, with the subsidence of the lipaemia, a gain in weight was noted. In contrast, those animals, in which there was no lipaemia, always showed a gain in weight. All of the rabbits continued to eat well after becoming diabetic. In addition, in the animals in which the elevation of serum lipids was most marked the degree of hyperglycaemia was greatest, although there was little difference in blood sugar level between the diabetic animals that showed only a moderate hyperlipaemia and those that showed none at all. From a consideration of these findings it appears that the elevation of serum lipids was due to a mobilization of fat from the body depots to meet the excessive demand for fat combustion caused by the disturbance in the regulation of carbohydrate metabolism. Our animals were never in diabetic coma and exhibited none of the signs of dehydration so that the factor of haemoconcentration(11) could hardly have played any part in the elevation of the serum lipids. At each determination not more than 10 cc of blood were drawn and it is known that the rabbit will tolerate the repeated loss of much larger quantities of blood than this without any change in the serum lipids(13).

It is interesting that the rise in total cholesterol was due much more to an increase in free cholesterol than to an elevation of cholesterol esters. This observation has already been made in the alloxan diabetic rabbit(2) and in the depancreatized dog fed raw pancreas before and after insulin deprivation(12). Consideration of the values obtained by the method of Forbes showed that from normal rabbit sera only a small proportion of lipid phosphorus and usually half, or less, of total and free cholesterol were "readily extractable", while all or nearly all of the neutral fat could be extracted by this method. In the diabetic animals, however, when the serum lipids were elevated, there was a marked increase in the proportion of "readily extractable" lipid phosphorus and the proportion of "readily extractable" total and free cholesterol was also increased. The neutral fat showed little change. It would appear, therefore, that in the normal rabbit the serum lipid phosphorus and cholesterol are to a large extent bound to the serum proteins; this has been suggested to be the case in normal human serum on the basis of electrophoretic studies(14). On the other hand, in the hyperlipaemia of alloxan diabetes in the rabbit the increase in lipid phosphorus and cholesterol appears to be largely in the fractions not closely bound to protein. Neutral fat did not appear to have any close association with protein, the irregularity of its behaviour being attributable to the errors inherent in its calculation.

*Summary.* In the early stages of alloxan diabetes in the rabbit there may or may not occur a transitory hyperlipaemia in which all of the serum lipid constituents, but especially the neutral fat, are elevated. In a few cases the hyperlipaemia may persist for an indefinite period of time. This elevation of serum lipids is apparently due to mobilization of fat from the tissue fat depots and is related to the severity of the diabetes. In the normal rabbit only a small proportion of the lipid phosphorus and the total and free cholesterol is "readily extractable"; in the hyperlipaemic serum of the alloxan diabetic rabbit a much greater proportion of these lipid constituents is "readily extractable". It is suggested that

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12. Gibbs, G. E., and Chaikoff, I. L., *Endocrinology,* 1941, v29, 885.

13. Dubach, R., and Hill, R. M., *J. Biol. Chem.*, 1946, v165, 521.

14. Blix, G., Tiselius, A., and Svensson, H., *J. Biol. Chem.*, 1941, v137, 485.

in the normal rabbit much of the lipid phosphorus and cholesterol of the serum is bound to the serum proteins, while in the hyperlipaemia of alloxan diabetes in the rabbit the increase of these lipids is principally in the fractions not closely associated with the serum proteins.

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# Increase in Glyceride Content of Brown Fat by Treatment with Adrenocorticotropin.* (17674)

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Although the important role played by adrenocorticotropin (ACTH) and the hormones of the adrenal cortex in the metabolism of carbohydrate and protein is well established, less is known concerning their effects on the metabolism of fat. However, it is becoming apparent that ACTH may modify the distribution of visible fat in the body. Thus, it has been demonstrated previously that ACTH may cause the disappearance of almost all of the fat of the tela subcutanea(1) while increasing the fat content of the liver (2,3) and marrow of the tibia(4). During the course of autopsies on animals treated with ACTH, it was observed that the brown fat of the interscapular gland had taken on somewhat the appearance of white adipose tissue. Therefore, we have undertaken to analyze this change with microscopic techniques.

*Methods.* The interscapular gland was studied from 10 adult male rats given 1 mg of ACTH daily for 21 days and fed a high fat diet by stomach tube and 3 castrated rats given the same dosage but a medium carbohydrate diet. For each treated rat there was 1 control. In addition 6 hypophysectomized rats were given 3 mg of ACTH daily for 21 days by *continuous* subcutaneous injection and fed *ad libitum.* In addition, brown fat in other locations was available for study in microscopic preparations made from many animals treated with ACTH as reported in our previous publications. These areas included the brown fat in the region of the kidneys, adrenal glands, superior mediastinum and neck. Samples of the interscapular gland were fixed in Bouin's fluid, 10% neutral formalin and chilled 80% alcohol and Carnoy's fluid. Lipids were stained with Sudan red, Sudan black B, Schiff reagent, Nile blue sulphate, subjected to the Schultz reaction, and also studied for auto-fluorescence and birefringence. The Gomori(5) procedure was used for alkaline phosphatase with sodium glycerophosphate and fructose diphosphate serving as substrates. Best's carmine and the Hotchkiss(6) periodic acid-leucofuchsin techniques were utilized for the demonstration of glycogen. Staining for cytoplasmic basophilia

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* Aided by a grant-in-aid from the Division of Research Grants and Fellowships, United States Public Health Service.

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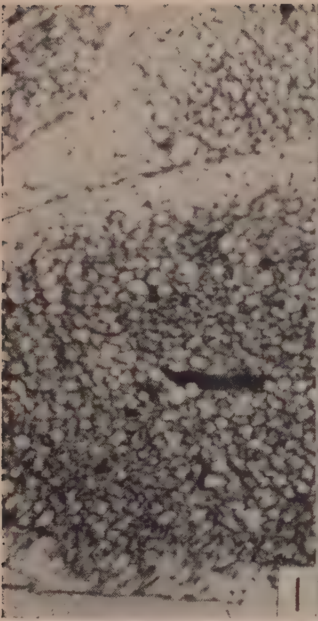

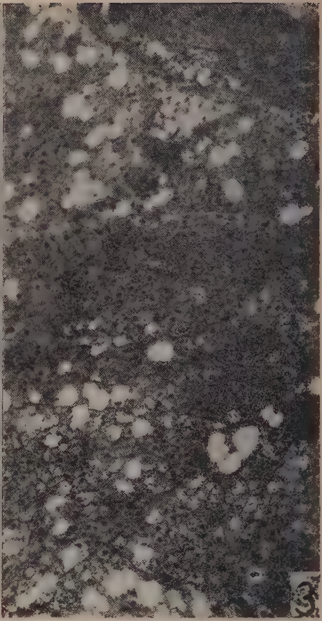

Fig. 1. Interscapular gland of castrated male rat, medium carbohydrate diet. ×110.

Fig. 2. Interscapular gland of castrated male rat, medium carbohydrate diet, 1 mg ACTH daily for 21 days. The vacuoles left by solution of glyceride are increased greatly. ×110.

Fig. 3. Interscapular gland of hypophysectomized male rat, fed *ad libitum*, 3 mg ACTH daily by continuous injection for 21 days. No increase in glyceride content has occurred. ×110.

was attempted with eosin and methylene blue (disodium phosphate-citric acid buffers).

*Observations.* The interscapular gland of the control animals was subdivided into lobules, with the typical brown fat tissue being concentrated around central blood vessels while a rim of cells similar to white fat in appearance formed a shell around the periphery of some lobules (Fig. 1.) Most of the central cells were multivacuolated after removal of lipid, were void of basophilia, and rarely revealed traces of glycogen. With the Gomori procedure little alkaline phosphatase activity was demonstrated with a glycerophosphate substrate, it being most evident in the nucleolus and occasionally in the blood vessel walls. With fructose di-phosphate as a substrate, phosphatase activity was more evident in both nuclei and cytoplasm. The lipid occurred in the form of multiple droplets and stained rose with Nile blue sulphate indicating that it was chiefly an unsaturated glyceride(7). The tissue in general gave a blue-green fluorescence which may have been due to the presence of some cholesterol or related substances in the cytoplasm of the brown fat cells. The cytoplasm of these cells stained intensely with the Schiff reagent (not preceded by treatment with mercuric chloride). The Schultz reaction for cholesterol was negative and, also, when thick frozen sections were extracted with warm chloroform, the typical blue color of the Lieberman-Burkhardt reaction failed to develop when acetic anhydride and sulfuric acid were added to the solution. However, observation of frozen sections of formalin-fixed tissue with the polarizing microscope revealed the presence of abundant birefringent material which was most concentrated in the central cells of the lobule. This birefringence disappeared completely when the slide was warmed, and upon cooling, a few spherocrystals giving the black cross of polarization appeared. These optical

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7. Lison, L., *Histochimie Animale,* Gauthier-Villars, Paris, 1936.

phenomena might be suggestive of the presence of cholesterol or its esters(7). Cramer(8) likewise experienced more difficulty in demonstrating cholesterol in the interscapular gland than in brown fat located in other regions of the body. It was apparent that the diet in itself modified the lipid content of brown adipose tissue. For example, this tissue contained less lipid in the controls of the castrated series fed a medium carbohydrate diet than in many of the intact series given a high fat diet.

Treatment of intact and castrated rats with adrenocorticotropin caused a regular increase in amount of sudanophilic lipid in the cells of brown fat as is illustrated by the vacuolation of the constituent cells from which the fat had been dissolved (Fig. 1 and 2). The smaller droplets of the normal cells had enlarged and coalesced to form one large droplet in many cells. In preparations from which fat had been removed, these cells had somewhat the appearance of white fat. It was concluded that this lipid was an unsaturated glyceride since it stained with a fat-soluble oxazone form of Nile blue sulfate and was generally isotropic. All of the other reactions characteristic of the controls remained in the compressed cytoplasm. The striking increase in vacuolation which characterized the brown fat from many other parts of the body after ACTH indicated that the increase in glyceride content was not limited to the brown fat of the interscapular gland. Likewise, the response was not conditioned entirely by the diet since evidence of increased fat content was secured regardless of whether rats were force-fed diets high in carbohydrate, fat or protein.

On the contrary, in the absence of the hypophysis, 3 times the dose of ACTH used in the preceding experiment failed to cause an increase in the glyceride content of brown fat (Fig. 3). In fact, this tissue contained less fat than in the control animals fed a high fat or medium carbohydrate diet by stomach tube.

Fawcett(9) has demonstrated recently that re-feeding of starved rats or treatment with insulin causes an increase in the amount of glycogen in brown adipose tissue. He pointed out further the probable importance of this glycogen in the synthesis of fat. In view of his work, it was of interest to find that ACTH caused no apparent increase in glycogen under the conditions of our experiments.

*Discussion.* It is not clear from our studies whether the ineffectiveness of ACTH in the hypophysectomized animal was due to the absence of an essential hypophyseal hormone or the unsatisfactory state of nutrition of these rats. Not only were they immobilized but, also, in eating *ad libitum*, they did not secure a normal food intake. In contrast to our findings, Fawcett and Jones(10) have reported that injection of ACTH into mice failed to increase the neutral fat content of interscapular adipose tissue. They also reported that ACTH prevented the loss of fat which occurred after hypophysectomy. Under the conditions of our experiments with rats, very large doses of ACTH were ineffective in preventing the loss of fat from the interscapular gland of hypophysectomized rats. Further, Selye and Timiras(11) reported recently that subjection of rats to cold resulted in depletion of the lipid content of the interscapular gland. At the present time, their findings cannot be reconciled with the effects which we observed after treatment with ACTH. However, they also observed the importance of the nutritional factor since starvation accentuated the loss of lipid from the interscapular gland due to exposure of rats to cold.

*Summary.* Treatment of intact or castrated rats with ACTH caused an increase in the glyceride content of brown adipose tissue. Treatment of hypophysectomized rats with 3 times the dose used above failed to maintain the fat content of the interscapular gland at a normal level.

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9. Fawcett, D. W., *Endocrinology*, 1948, v42, 454.

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# Cross-Neutralization Tests with Coxsackie Viruses. (17675)

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The variable clinical features presented by the patients admitted to the Parkland City-County Hospital at Dallas with an initial diagnosis of poliomyelitis during the 1949 epidemic suggested multiple etiologic agents. Studies were undertaken on materials from selected patients to determine whether agents other than poliomyelitis virus were causative factors in this outbreak. This report is concerned with a study of the serological relationship between 2 strains of Coxsackie virus isolated in this laboratory and those recovered by other investigators(1-4). These 2 strains of Coxsackie virus (Dallas—M.B. and Bonham—N.V.), isolated from stools of patients with paralytic infection, produced paralysis and myositis in infant mice. The patients from whom they were obtained showed residual muscular weakness four months after onset of the illness, and neutralizing antibodies for the homologous strain have been demonstrated in their sera. A significant rise in neutralizing antibody titer was also demonstrated in serum specimens from a non-paralytic patient who had been in close contact with one of these patients prior to illness. Included in this serological analysis is another strain isolated in Texas, the B.J. strain, kindly provided by Dr. J. V. Irons. The Texas 1-2 strain, recovered from flies trapped in the Rio Grande Valley in 1947, and the Connecticut No. 5 strain were provided by Dr. J. L. Melnick. Coxsackie strains T.T. (type 1), Fl. (type 2) and J.Ol. (type 3) were supplied by Dr. Gilbert Dalldorf.

Immune sera were prepared in guinea pigs for the Dallas—M.B., Connecticut No. 5 and Coxsackie T.T. (type 1) strains by intraperitoneal injections of 10% infected mouse brain tissue. Neutralization tests were carried out by mixing equal amounts of immune serum with the supernatant fluid from a lightly centrifuged 10% suspension of brain tissue from infected mice. The mixture was allowed to stand at room temperature for one hour prior to intra-abdominal injection into 1- to 3-day-old suckling mice. Each mouse was inoculated with 0.03 ml of the mixture and all animals were observed for a period of 12 days. The results of these neutralization tests are shown in the accompanying table.

It is evident from the results presented in this table that the 2 strains isolated in this laboratory (Dallas—M.B. and Bonham—N.V.) are serologically identical and appear to be immunologically related to the Texas 1-2 strain. The Connecticut No. 5 strain was neutralized only by the homologous antiserum and showed no relationship to any of the other strains studied. Failure of the animals to develop paralysis, rather than survival, was used as the criterion of neutralization in the case of this strain, as many of the animals showing paralysis survived longer than the observation period of 12 days. The Connecticut No. 5 strain should probably be classified among Dalldorf's recently designated Group B strains due to its peculiar behavior in the experimental animal(5,6). The two strains recovered in this laboratory differ also from the strains received from Dalldorf as shown by the fact that type 1 serum failed to neutralize either the Dallas—M.B. or Bonham—N.V. strains, and by the fact that the serum prepared against the Dallas—M.B. strain failed to neutralize the Coxsackie types 1, 2 and 3. In a previous report, Sickles and Dalldorf(6,7) have shown that the types

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TABLE I.
Cross-Neutralization Tests with Coxsackie Viruses.

| | | Immune serum | | |
|---|---|---|---|---|
| Virus strain[*] | Controls | Dallas—M.B. | Conn. No.5 | Coxsackie—T.T. (type 1) |
| Dallas—M.B. | 2,2,2,3,3[†] | S,S,S,S | 4,4,4,4 | 3,3,3,4 |
| Bonham—N.V. | 2,2,2,2,2,3,3,3 | S,S,S,S,S | 3,4,4,4 | 4,4,4,5,5 |
| Texas 1-2 | 3,3,3,3,4,4,4 | S,S,S,S | — | 3,4,4,4,4 |
| Conn. No. 5 | 5,5,5,7,7,7,7 | 5,5,5,5 | S,S,S,S | 3,3,3,5,5,5,5 |
| Coxsackie—T.T. (type 1) | 3,3,3,3,3,3,3 | 3,3,3,3 | 3,3,3,3 | S,S,S,S,S |
| ” —Fl. (type 2) | 2,2,2,2,3 | 3,3,3,3,3 | 3,3,3,3,3 | — |
| ” —J.Ol. (type 3) | 2,2,2,2,3 | 2,2,2,2,3 | 2,2,2,3,3 | — |
| B.J. | 5,5,7,7,7 | 7,7,7,7 | 5,5,7,7 | — |

[*] 10% infected mouse brain suspension.
[†] Figures indicate days of death or paralysis.
S—Animal survived.
— Tests not done.

1, 2 and 3 are serologically distinct. Dalldorf(6) has further indicated that the Texas 1-2 strain of Melnick differs serologically from the strains which he has designated types 1, 2 and 3. Melnick and associates(3) have reported that the Connecticut No. 5 and Texas 1-2 strains are unrelated.

Thus it appears that at least 5 serological types of Coxsackie virus have been encountered. Whether the B.J. strain represents another serological type or is a member of the Coxsackie types 1, 2 or 3 has not been determined.

The observation that 2 of the strains (Dallas—M.B. and Bonham—N.V.) isolated in this laboratory are similar to the strain isolated by Melnick from flies collected in the Rio Grande Valley in 1947(3) and the relationship of the latter to a strain isolated from sewage collected in North Carolina(3) may have epidemiological significance. To our knowledge human infection with this type has not been previously reported.

*Summary.* A serological comparison of 2 virus strains recently isolated from human paralytic infections and other viruses of the Coxsackie family is presented. The results indicate that at least 5 serological types of Coxsackie virus have been encountered. To our knowledge human infection with the type isolated in this laboratory has not been previously reported.

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## Complement Fixation with a Coxsackie Virus. (17676)

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The need for a rapid and simple method of diagnosis in Coxsackie virus infections prompted an investigation of the complement-fixing capacity of virus suspensions and specific immune serums. Because of the widespread occurrence of neutralizing antibodies in presumably normal persons, the neutralization test as previously described(1,2) and as done in this laboratory may prove to be of limited value in the diagnosis of Coxsackie virus infections. In addition, the procedure is time-consuming and costly. Immune guinea

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2. Melnick, J. L., Shaw, E. W., and Curnen, E. C., Proc. Soc. Exp. Biol. and Med., 1949, v71, 344.

TABLE I.
Complement Fixation by Immune Guinea Pig Serum and Coxsackie Virus Infected Tissue Antigens.

| | | Antigen dilutions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Virus antigen | | | | | Control antigen | | | |
| Nature of antigen | Dilution of immune guinea pig serum | 8 | 16 | 32 | 64 | 128 | 4 | 8 | 16 | 32 |
| Crude suspension | 1:4 | 4 | 4 | 4 | 2 | 0 | 4 | 3 | 2 | 1 |
| | 1:16 | 4 | 4 | 1 | 0 | 0 | —* | 0 | 0 | 0 |
| | 1:32 | 4 | 4 | 0 | 0 | | — | 0 | 0 | |
| | 1:64 | 4 | 1 | 0 | 0 | | — | 0 | | |
| | 1:128 | 2 | 1 | 0 | | | — | 0 | | |
| | 1:256 | 1 | 0 | 0 | | | — | 0 | | |
| Centrifuged 2500 rpm 30 min. | 1:4 | 4 | 4 | 4 | 2 | 0 | 3 | 1 | 0 | 0 |
| | 1:16 | 4 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | |
| | 1:32 | 4 | 1 | 0 | 0 | | 0 | 0 | | |
| | 1:64 | 4 | 1 | 0 | 0 | | 0 | 0 | | |
| | 1:128 | 0 | 0 | 0 | | | 0 | 0 | | |
| | 1:256 | 0 | 0 | | | | 0 | 0 | | |
| Centrifuged 5000 rpm 30 min. | 1:4 | 4 | 4 | 2 | 1 | 0 | 2 | 0 | 0 | 0 |
| | 1:16 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | |
| | 1:32 | 4 | 1 | 0 | | | 0 | 0 | | |
| | 1:64 | 0 | 0 | | | | 0 | 0 | | |
| | 1:128 | 0 | 0 | | | | 0 | 0 | | |
| | 1:256 | 0 | 0 | | | | 0 | 0 | | |

4 = Complete fixation.
3, 2, and 1 = proportionately lesser fixation.
0 = Complete hemolysis.
* Reading of hemolysis not accurate due to turbidity of crude antigen in this dilution.

pig serum for the Dallas—M.B. strain(3) was used for the initial experiments. Complement fixation tests with two types of antigens have been done. In the first attempts, unsuccessful results were obtained when crude suspensions of brain tissue from infant mice infected with the Dallas—M.B. strain was used as antigen. Following the report of Gifford and Dalldorf(4) that the virus content of paralyzed muscle of infected mice is many times greater than brain material, the legs of mice infected with this strain were harvested and ground with alundum as a 33% suspension in saline. The mixture was centrifuged at 2500 r.p.m. for 2 minutes and the supernatant fluid was tested for its suitability as antigen in the complement fixation test. Positive results were obtained with this preparation, but non-specific fixation occurred as shown by tests with tissue extracts prepared from normal infant mice using the same procedure as for virus antigen. The results led to a third series of experiments with centrifuged virus and control antigens treated in an identical manner. These antigens were prepared from crude muscle tissue suspensions as described above and further treated as follows: 1. Supernatant fluid from ground muscle tissue centrifuged at 2500 r.p.m. for 2 minutes. 2. Supernatant fluid from the same material centrifuged at 2500 r.p.m. for 30 minutes. 3. Supernatant fluid from same material centrifuged at 5000 r.p.m. for 30 minutes.

In the test, the immune guinea pig serum was inactivated at 56°C for 30 minutes and diluted 1:4, 1:16, 1:32, 1:64, 1:128 and 1:256. Virus antigen dilutions of 1:8, 1:16, 1:32, 1:64 and 1:128, and normal tissue extracts (control antigen) dilutions of 1:4, 1:8, 1:16 and 1:32 were prepared. Each dilution of immune serum was tested with each of the dilutions of both virus and control antigens. The immune serum (0.2 ml) was mixed with an equal amount of the appropriate dilution of the antigen and 0.2 ml of complement con-

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3. Sulkin, S. E., Manire, G. P., and Farmer, T. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 340.

4. Gifford, R., and Dalldorf, G., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 589.

taining 2 exact units. The tubes were incubated in a water bath at 37°C for 2 hours and 0.4 ml of a mixture containing equal parts of 2% washed sheep erythrocytes and hemolysin (2 units per 0.2 ml) was added to each of the tubes. The tests were read after further incubation for 30 minutes. Complete fixation was recorded as 4+ and complete hemolysis as 0. The usual controls were included. The results of these tests are shown in the accompanying table.

An analysis of the results would indicate that antigens prepared from infected mouse muscle tissue give specific fixation of complement in the presence of homologus guinea pig immune serum. These sera failed to fix complement in the presence of comparable dilutions of antigens prepared from normal mouse muscle tissues. Centrifugation of crude antigens further increased the specificity of the test as shown by the comparable titers obtained with the virus antigen after centrifugation at 2500 r.p.m., whereas non-specific fixation was virtually eliminated. Further centrifugation at 5000 r.p.m. did not improve the antigen. Additional studies on the use of other purification procedures and on the reactivity of antigens prepared with other serologic types are in progress.

Preliminary observations on the use of this procedure in analyzing the sera from patients from whom the virus has been isolated, patients suspected of infection with this virus, and from presumably normal individuals, indicate that the complement fixation test may be of value in the study of Coxsackie infection in the human.

*Summary.* The technique of a complement fixation test which may prove of value in the study of Coxsackie virus infection in man is described.

*Addendum.* Since this manuscript was submitted for publication, Casals *et al.*, Howitt and Benefield, and Melnick *et al.* have reported complement fixation with strains of Coxsackie virus.

The authors are indebted to Miss Rae Allen for technical assistance.

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## Homologous Transplantation of Fetal Endocrine Tissue to Adult Non-Related Host.* (17677)

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This report concerns itself with the surgical transplantation of endocrine tissues to normal animals. Previous investigators have made either autographs [Halstead(1), Ingle (2)] of the parathyroids and the adrenals, or syngenesiographs [Pomerat(3), Robertson(4) and Turner(5)] of the adrenal and ovary. Review of the literature reveals no reports of successful transplantation of endocrine tissue to animals of the same species which are not closely related—a homologous graft. Greene(6) has demonstrated that embryonic tissues, like cancer tissue, possess autonomy and may be transplanted to the anterior chamber of the eye of a foreign host. The purpose of this paper is to report the successful homologous transplantation of embryonic adrenal, ovary, testes and thyroid to the anterior chamber of the eye of adult hosts.

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* This investigation was supported by Grant No. C-867 from the National Cancer Institute and the Surgical Research Fund of the Buffalo General Hospital.

† Post-doctorate Research Fellow, National Cancer Institute, United States Public Health Service.

1. Halstead, W. S., *J. Exp. Med.*, 1909, v11, 175.
2. Ingle, D. J., and Higgins, G. M., *Endocrinology*, 1938, v22, 458.
3. Pomerat, C. M., Breckenridge, C. G., and Gordon, L., *Endocrinology*, 1944, v34, 60.
4. Robertson, G. C., PROC. SOC. EXP. BIOL. AND MED., 1945, v59, 30.
5. Turner, C. D., *Anat. Rec.*, 1939, v73, 145.
6. Greene, H. S. N., *Cancer Research*, 1943, v3, 809.

*Methods.* The feti were removed aseptically by cesarean section and the endocrine tissues were removed with the aid of the dissecting microscope. The tissues were transplanted, under local anesthesia, to the anterior chamber of the eye of non-related adult hosts within one to 5 hours after removal. The rabbit fetal tissues were removed between the 18th and 22nd day of gestation, and the guinea pig and dog at approximately the 42nd day. All material for histological study was fixed in 10% formaldehyde and stained with hematoxylin-eosin. The study was controlled in the following manner. At the time of transplantation, a piece of fetal endocrine tissue was fixed for histological study, its size, diameter and cellular maturity being noted. The transplanted tissue when removed at a later date is compared with the control section for growth in size, cellular maturity and differentiation. No transplant was classified as a "take" unless there was histological evidence of growth for 30 days or longer.

TABLE I.
Rabbit Fetal Endocrine Tissue Transplanted to the Anterior Chamber of the Eye of Normal Rabbits, Other Than the Mother.

(A) 21 gonads transplanted:
3 growing and surviving between 6 to 7 wks.
3 ” ” ” ” 9 to 10 ”
1 ” ” ” at end of 108 days.
2 regressed between 2 and 3 wks.
4 ” ” 4 ” 5 ”
1 ” ” 6 ” 7 ”
7 initial failures.
Summary: 7 continued to grow 42 days or longer.

(B) 13 adrenals transplanted:
2 growing and surviving between 2 and 3 wks.
1 ” ” ” ” 6 to 7 ”
3 ” ” ” ” 9 ” 10 ”
1 ” ” ” ” 10 ” 11 ”
1 ” ” ” ” 13 ” 14 ”
2 regressed between 2 to 3 wks.
1 ” ” 3 ” 4 ”
1 ” ” 8 ” 9 ”
1 initial failure.
Summary: 6 continued to grow 35 days or longer.

(C) 12 thyroids were transplanted:
2 growing and surviving between 4 to 5 wks.
1 ” ” ” ” 6 ” 7 ”
3 ” ” ” ” 9 ” 10 ”
1 ” ” ” ” 13 ” 14 ”
1 regressed between 2 to 3 wks.
1 ” ” 4 ” 5 ”
3 initial failures.
Summary: 5 continued to grow 42 days or longer.

TABLE II.
Guinea Pig Fetal Endocrine Tissue Transplanted to the Anterior Chamber of the Eye of Normal Guinea Pigs, Other Than the Mother.

(A) 15 gonads were transplanted:
2 growing and surviving between 4 to 5 wks.
2 ” ” ” ” 5 ” 7 ”
2 ” ” ” ” 8 ” 9 ”
2 ” ” ” ” 9 ” 10 ”
1 ” ” ” ” 10 ” 11 ”
6 initial failures.
Summary: 7 continued to grow 35 days or longer.

(B) 15 adrenals were transplanted:
2 growing and surviving between 4 to 5 wks.
2 ” ” ” ” 5 ” 6 ”
1 ” ” ” ” 6 ” 7 ”
1 ” ” ” ” 8 ” 9 ”
2 ” ” ” ” 10 ” 11 ”
1 ” ” ” ” 14 ” 15 ”
1 died, transplant survived between 2 to 3 wks.
5 initial failures.
Summary: 5 continued to grow longer than 42 days.

(C) 15 thyroid glands were transplanted:
3 died between 2 to 3 wks, and surviving.
3 growing and surviving between 4 to 5 wks.
2 ” ” ” ” 5 ” 7 ”
3 ” ” ” ” 8 ” 13 ”
1 still living and growing at end of 150 days.
3 initial failures.
Summary: 6 continued to grow longer than 42 days.

*Results and discussion.* Detailed results are listed in Tables I, II and III. There has been a 50 to 60% survival and continuous growth in the homologous embryonic transplants of the adrenal, ovary, testes and thyroid to the adult guinea pig and rabbit. The percentage of "takes" in the dog has been less. We believe this to be primarily a technical problem and are investigating methods to overcome the difficulty. In the fetal thyroids at the time of transplantation the tiny immature follicles are scant with colloid if any at all is present; the transplants removed 30 to 180 days later had increased more than 30 times in volume, there was cellular maturity of the follicles, and each follicle con-

TABLE III.
Dog Fetal Endocrine Tissues Transplanted to the Anterior Chamber of the Eye of Normal Dogs and Puppies.

(A) 9 gonads were transplanted:
  1 grew for approximately 60 days and regressed.
  2 abscesses.
  6 initial failures.

(B) 11 adrenal transplants:
  4 "Takes"—2 were growing and surviving between 7 to 8 weeks.
  5 initial failures.
(C) 8 thyroid transplants:
  2 growing and surviving at the end of 30 days.
  6 initial failures.

tained colloid. The fetal ovaries after transplantation show maturity and follicular development, and in the case of the rabbit, the transplant will give a positive Friedman reaction. Rabbit fetal testes removed eighty-five days after transplantation show spermatids. There is increased differentiation of the various zones of the adrenal cortex as well as cellular growth and multiplication of cells. The medulla does not survive. Pancreas transplants removed 30 to 120 days later reveal well formed ducts lined with low cuboidal epithelium, but the Islets of Langerhans have all regressed. We are investigating methods of increasing the percentage of survival by applying Halstead's Law of Endocrine Deficiency either at the time of transplantation or soon thereafter. We may state that the percentage of "takes" of the ovary and testes has increased from 60% to 90%. This observation will be reported at a later date upon a larger series which is now under investigation. In the case of the adrenal and thyroids, our best growths and survivals have been in those animals which had the corresponding endocrine organs removed. In our laboratory thyroidectomized guinea pigs have homologous fetal thyroid transplants which have been growing for over 10 months. We have also been investigating sites for transplantation other than the anterior chamber of the eye in order to transplant fetal endocrine tissue clinically. In only a few cases have we been successful, this site being the kidney, with the adrenal transplant.

*Summary*. (1) Embryonic adrenal, ovary, thyroid and testes have been homologously transplanted to the anterior chamber of the eye of normal dogs, guinea pigs and rabbits with a 50 to 60% survival beyond six weeks in the latter 2 species of normal animals. (2) In transplants of pancreas, only the ducts survived. (3) The best growth occurred in those animals in which the corresponding endocrine organ was removed.

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## The Preparation of Secretin. (17678)

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Several methods have been described for extraction and purification of secretin but unfortunately these have in general proved to be cumbersome, time-consuming, or unsuitable for producing secretin economically in more than small quantities. The present report describes a simplified procedure for preparation of a potent secretin which has been given intravenously to more than 150 human subjects without evidence of untoward reactions. The potency of various fractions was tested on dogs by an assay procedure described elsewhere(1) and expressed in terms of the clinical unit established by Hammersten and coworkers(2) which, as was shown by Greengard and Stein(3), is identical with the Ivy

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2. Hammersten, E., Agren, G., and Lagerlof, H., *Acta Med. Scand.*, 1937, v92, 256.

3. Greengard, H., and Stein, I. F., Proc. Soc. Exp. Biol. and Med., 1941, v46, 149.

dog threshold unit(4). Tests have shown the following substances to be either absent from the secretin preparations or present in barely detectable concentrations: vaso-depressor substances (blood pressure method on atropinized cat); pancreozymin (determinations of concentration of trypsin(5) and amylase(6) of the pancreatic juice); cholecystokinin (unanesthetized dog method(7)); hepatocrinin (unanesthetized cholecystectomized dog method(8)); enterocrinin* (procedure of Nasset (9)) and hypoglycemic substances (blood sugar determinations by Folin-Wu method).

*Preparation of Secretin.* The most suitable starting point was found to be preparation of a salt cake as described by Voegtlin, Greengard and Ivy(10). This consists of soaking for 30 minutes the everted upper 6 feet of hog intestine in ice-cold 0.13 N HCl, 200 ml per meter of intestine. Constant stirring is avoided since this results in extraction of large amounts of inert material from which the secretin is subsequently separated with difficulty. The intestine is removed and sodium chloride is added with constant stirring to the acid solution to 32% concentration. Following 15 to 24 hours storage in the refrigerator (5°C) the supernatant fluid is decanted and the precipitate is partially dried by *pressure filtration* until it contains not more than 40% moisture. The filtrate contains vasodepressor substances and drying the salt cake by evaporation only reduces the moisture content without removing them. To the salt cake are added 5 parts by weight of absolute acetone-free methanol. After storage at 5°C for 12 hours the methanol is separated by filtration. The residue is reextracted for several hours in the cold with 2.5 parts of methanol and the methanol filtrates combined if studies on distribution and yield of secretin are undertaken. Large quantities of secretin however may be prepared most economically by only a single methanol extraction. To the methanol filtrates are added exactly 3 volumes of absolute acetone. Addition of more acetone than this yields a greater precipitate but the additional material so obtained is inert as a pancreatic stimulant. After not more than 24 hours storage in the refrigerator the acetone precipitate is collected by filtration and dried with acetone and ether. The precipitate is agitated in cold saturated sodium chloride solution, 50 ml per g, and the material remaining insoluble after two hours is recovered and dried with acetone and ether.

The precipitate is suspended without agitation in approximately 100 times its weight of distilled water which has been acidified with 1 part of glacial acetic acid. After storage for 12 hours in the refrigerator the insoluble material (often found floating on the surface) is removed by filtration in the cold. To the cold filtrate enough cold solution of 100% trichloracetic acid is added to make a final concentration of 10%. Maximum precipitation occurs during storage in the refrigerator for 24 to 72 hours. The fine precipitate is collected by centrifugation at low speed and washed 5 times with absolute acetone, 3 times with absolute ethyl ether, and dried over calcium chloride. The dry precipitate is agitated for 6 hours in normal butanol, 100 ml per g precipitate, in a water bath at 40°C. The insoluble material obtained by centrifugation is retained and washed 3 times with absolute acetone and twice with absolute ethyl ether.

After determining the potency as described elsewhere(1), the active material is dissolved in distilled water to a concentration of 20 clinical units per ml. The aqueous solution is measured into vials or ampules of 5 ml capacity and reduced to the dry state by freezing and lyophilization in the usual manner.† The vials, each containing a stand-

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4. Greengard, H., and Ivy, A. C., *Am. J. Physiol.*, 1938, v124, 427.

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7. Snape, W. J., Friedman, M. H. F., and Thomas, J. E., *Gastroenterology*, 1948, v10, 496.

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* We are indebted to Professor E. S. Nasset who kindly determined the effect of certain preparations on the secretion of the small intestine.

10. Voegtlin, W. L., Greengard, H., and Ivy, A. C., *Am. J. Physiol.*, 1934, v110, 198.

TABLE I.
Effect of Intravenous Injection of Secretin at 10-minute Intervals on the Pancreatic Secretion in Unanesthetized Dog (Temporary Intubation of Pancreatic Duct *in Situ* via Duodenal Fistula).

| Dog No. | Date, 1946 | No. of samples | Vol. of 10-min. samples Max, ml | Min, ml | Avg, ml | N, mg/cc | Trypsin, K × $10^{-3}$ |
|---|---|---|---|---|---|---|---|
| 1-44 | 1/31 | 4 | 4.8 | 4.2 | 4.55 | 1.6 | 100 |
| | 2/18 | 4 | 4.7 | 2.4 | 3.7 | 1.9 | 100 |
| | 7/17 | 4 | 3.3 | 2.5 | 3.0 | 1.6 | 80 |
| 1-45 | 1/25 | 2 | 6.0 | 5.7 | 5.85 | 1.5 | 117 |
| | 2/13 | 2 | 5.0 | 4.7 | 4.85 | 1.0 | 97 |
| | 3/21 | 3 | 5.4 | 5.0 | 5.2 | 1.6 | 125 |
| 1-46 | 2/14 | 3 | 3.0 | 2.5 | 2.8 | 2.0 | 120 |
| | 5/ 1 | 3 | 6.6 | 5.1 | 5.7 | 1.5 | 120 |
| | 14 | 6 | 8.6 | 1.5 | 5.75 | 1.5 | 105 |
| 2-46 | 7/12 | 3 | 4.0 | 2.8 | 3.6 | 1.6 | 77 |
| | 18 | 4 | 6.4 | 4.6 | 5.5 | 1.7 | 112 |
| | 22 | 4 | 6.2 | 3.1 | 4.8 | 1.3 | 90 |

ard amount of secretin, are then sealed and stored. The lyophilized secretin is readily soluble in distilled water. No detectable loss in potency of the material sealed in the ampules has been found after one year's storage at room temperature.

Single, multiple(11) and continuous(12) injections of this preparation of secretin in doses up to 18 units per kilo body weight have been made in unanesthetized dogs without untoward reactions. Single, double and triple hourly injections of this secretin in 1.1 units per kilo body weight have been made in over 150 human subjects without evidence of cardiovascular, respiratory, or anaphylactoid reactions(13).

In Tables I and II are summarized typical results obtained in the dog and man following intravenous administration of secretin prepared as described above. The pancreatic function tests in man were carried out by the use of a double lumen tube with constant aspiration of the gastric and duodenal contents (as described by Lagerlof(14)). Pancreatic juice in the dog was obtained by direct intubation of the pancreatic duct in the unanesthetized animal as described by Hart and Thomas(15). The low concentrations of enzymes and nitrogen in the pancreatic juice secreted in response to secretin injection offer evidence of the absence of pancreozymin or other ecbolic agents from the secretin preparation.

*Discussion.* The most potent secretin preparation hitherto described is the secretin picrolonate of Hammersten, Agren *et al.*(16). Its potency of 1 cat unit per .004 mg would correspond to 16 clinical units per mg. Our best preparations assayed 12 clinical units per mg but the usual product ranged from 6 to 8 units. Since in the picrolonate the secretin appears to be combined with variable amounts of picrolonic acid, it is impossible to make an exact comparison between the two preparations. Further fractionation of our preparations was found to increase the potency

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† Lyophilization for the purpose of increasing solubility was suggested by Dr. F. W. Bernhart to whom we are greatly indebted for carrying out this stage in the procedure and for much invaluable assistance. Financial assistance from Wyeth Incorporated is acknowledged.

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12. Thomas, J. E., and Crider, J. O., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 27.

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15. Hart, W. M., and Thomas, J. E., *Gastroenterology,* 1945, v4, 409.

16. Hammersten, E., Agren, G., Hammersten, H., and Wilancer, O., *Biochem.,* 1933, v264, 275

TABLE II.
Effect of Secretin on Pancreatic Secretion in Man.

| Subject | Fasting duodenal content, 20-min. sample | | Following secretin | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 20 min. | | 40 min. | | 60 min. | |
| | Vol., ml | Trypsin, K × 10-3 | Vol., ml | Trypsin, K × 10-3 | Vol., ml | Trypsin, K × 10-3 | Vol., ml | Trypsin, K × 10-3 |
| Student | 7 | 60 | 82 | 110 | 61 | 25 | 50 | 10 |
| Psycho-neurosis | 4 | 20 | 51 | 162 | 42 | 70 | 35 | 52 |
| Chronic ulcerative colitis | — | 120 | 60 | 120 | 32 | 75 | 14 | 50 |
| Duodenal ulcer | 14 | 45 | 111 | 100 | 48 | 68 | 50 | 20 |
| Chronic pancreatitis | 15 | 0 | 90 | 0 | 51 | 0 | 43 | 0 |
| Chronic pancreatitis advanced | 4 | 0 | 10 | 0 | 10 | 0 | 4 | 0 |

but only at a tremendous and uneconomical loss in yield. In view of the recent work of Greengard, Wolfrom and Ness(17), showing that crystalline secretin picrolonate is indistinguishable by crystallographic methods from crystalline pyridine picrolonate, it is questionable whether attempts at crystallization of secretin by similar procedures would prove profitable.

Secretin made as described here proved suitable for intravenous administration in man. It was free of vasodepressor and pyrogenic substances and apparently non-antigenic. Cholecystokinin, enterocrinin, pancreaozymin, and hypoglycemic substances were not present in measurable amounts. It is of importance to note that the procedure is relatively simple and that reproducible results have been obtained consistently by the authors in this laboratory as well as by investigators in another institution(18).

*Summary.* A method for the preparation of a potent secretin suitable for intravenous administration in man is described.

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## The *In Vitro* Conversion of C14-Labeled Glucose to Fatty Acids.* (17679)

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Although the importance of lipogenesis in carbohydrate utilization has been recognized for some time, it was Stetten and Boxer who first called attention to the quantitative significance of this conversion in the normal animal(1). With the aid of deuterium as a labeling agent, they showed that, in the rat fed a high carbohydrate diet, at least 10 times as much of the dietary glucose is used to synthesize fatty acids as is used in the synthesis of glycogen. More recently, lipogenesis was studied with $C^{14}$-labeled glucose by Masoro *et al.*(2). In mice that had been maintained for some time on a high carbohydrate diet, 10-15 per cent of the ingested glucose-$C^{14}$ was recovered as fatty acids in 24 hours, and 12 and 16% in 48 hours. These recoveries were observed in mice whose weights had remained constant for a 2-week

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* Aided by grant from the American Cancer Society as recommended by the Committee on Growth of the National Research Council.

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TABLE I.

The Conversion of $C^{14}$-Glucose to Fatty Acids by Surviving Liver, Kidney, and Diaphragm Prepared from Rats Fed a High Carbohydrate Diet.

Each incubation flask contained 350-430 mg of tissue, 4.5 cc of Krebs-bicarbonate buffer, and 0.5 cc of a saline solution containing $C^{14}$-labeled glucose. The period of incubation was 3 hours at 37.5°. The contents of 2 or more flasks were pooled for analysis.

| | | | | | % of added glucose-$C^{14}$ recovered as | |
|---|---|---|---|---|---|---|
| Exp. | Tissue | Avg amt of tissue per flask, mg | Initial glucose conc. of medium,§ mg % | No. of flasks pooled for analysis | $CO_2$ per g tissue | Fatty acids, per g tissue |
| 1* | Liver | 313 | 422 | 3 | | .40 |
| | Kidney | 340 | 350 | 9 | | .04 |
| | Diaphragm | 370 | 350 | 4 | | .05 |
| 2† | Liver | 310 | 432 | 5 | | .44 |
| | Kidney | 314 | 432 | 5 | | .04 |
| | Diaphragm | 337 | 432 | 5 | | .02 |
| 3‡ | Liver | 513 | 418 | 2 | 5.8 | .56 |
| | Diaphragm | 405 | 418 | 2 | 2.5 | .04 |
| | Skeletal muscle | 765 | 418 | 2 | 1.2 | .01 |
| 4‡ | Liver | 496 | 392 | 2 | 4.7 | .81 |
| | Diaphragm | 510 | 392 | 2 | 3.0 | .17 |

* Liver slices were prepared from a single rat, whereas the amounts of kidney and diaphragm slices recorded above were obtained from 3 rats.

† The amount of each tissue incubated was obtained from a pool containing slices from 4 rats.

‡ All tissues used in this experiment were obtained from a single rat.

§ Glucose concentration of medium before addition of slices.

period. There can no longer be any doubt, from these findings as well as from the observations of Stetten, that lipogenesis from carbohydrate does occur in rats and mice maintained in the steady state.

$C^{14}$-labeled glucose has been used in the present investigation to study lipogenesis by surviving slices of liver, kidney, and muscle that were prepared from rats that had been fed a high carbohydrate diet. When these slices were incubated in a Ringer-bicarbonate medium containing 0.4 per cent of $C^{14}$-labeled glucose, significant amounts of the $C^{14}$ were converted to fatty acids.

*Experimental. Animals and tissues.* Rats were fed, for 2 or more weeks, a high carbohydrate diet containing 60% dextrose, 22% casein, 6% cellu-flour, 6% brewer's yeast, and 6% salt mixture(3). The rats also received, twice weekly, several drops of a fish oil containing 400 A.O.A.C. chick units of vitamin D and 3000 U.S.P. units of vitamin A per g. The rats, which weighed from 150 to 250 g, were sacrificed by fracture of the cervical vertebrae, and the required tissues were rapidly removed. Slices of liver and kidney were prepared freehand with a thin razor blade. The diaphragm was trimmed to remove the central tendon and extraneous tissue. The tissues were collected in a Petri dish containing cold Ringer-bicarbonate solution. Before their transfer to the incubation flasks, they were blotted free of excess moisture, and weighed.

*Radioactive Glucose.* The radioactive glucose was prepared photosynthetically according to the method of Putnam *et al.*(4). It was shown to be uniformly labeled with $C^{14}$(5). We are indebted to Dr. W. Z. Hassid and Mr. S. Abraham for the sample of labeled glucose used in this study.

*Incubation procedure.* The Ringer-bicarbonate solution was prepared according to Krebs and Henseleit(6), and adjusted to pH

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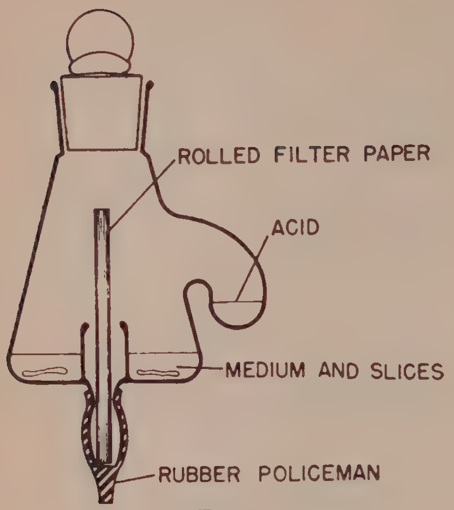

FIG. 1.
Incubation flask.

7.4 just before use. To each flask was added 4.5 cc of this buffer and 0.5 cc of a saline solution containing 4% labeled glucose. The specific activity of the added glucose was 1 x $10^4$ counts per minute per mg of glucose.

The flasks were gassed with a mixture consisting of 95% $O_2$ and 5% $CO_2$. The flasks were then placed in a constant temperature water bath maintained at 37.5°, and gently shaken during the 3 hours of incubation.

*Collection of $C^{14}O_2$.* In the first 2 experiments (Table I), 50-cc, glass-stoppered Erlenmeyer flasks were used for incubation. At the end of the experiment the stopper was removed and 1 cc of 1N HCl was added to stop the reaction. This procedure did not, of course, permit collection of the $CO_2$ within the flask.

In experiment 3 and 4, incubation of the tissue slices was carried out in Erlenmeyer flasks modified, as shown in Fig. 1, to permit collection of the $CO_2$. An amount of 5N $H_2SO_4$ (usually 0.2 cc) sufficient to stop the reaction and to liberate the $CO_2$ from the medium was placed in the sidearm. The center well extended through the bottom of the flask and was sealed with a rubber policeman. A roll of No. 50 Whatman filter paper was placed in the center well. Five minutes before the end of the experiment, approximately one cc of 30% KOH was injected through the rubber policeman into the well with the aid of a syringe. The alkali was rapidly taken up by the filter paper. At the end of the experiment the flask was tilted so as to transfer the acid from the sidearm to the medium. The flask was allowed to stand for 30 minutes to permit absorption of the $CO_2$ by the alkali. The filter paper was then removed from the center well and placed in a 50-ml volumetric flask. The center well was washed 5 times with distilled water and the washings were transferred to the volumetric flask. Aliquots were removed for the determination of $C^{14}$ according to the method of Entenman *et al.*(7). The efficiency of this method for collecting $CO_2$ was tested as follows. Five cc. of a solution of $Na_2CO_3$ labeled with $Na_2C^{14}O_3$ were placed in the main compartment of the flask, and 0.2 cc of 5N $H_2SO_4$ in the sidearm. One cc of 30% KOH was then injected into the center well, the flask was tilted, and the acid mixed with the carbonate solution. The flask was allowed to stand for 30 minutes. In several experiments of this type, 97-99% of the added $C^{14}$ was recovered.

*Analysis of Flask Contents.* After incubation, the acidified contents of several flasks were combined for analysis. The combined contents were filtered. The tissue on the filter paper was then thoroughly washed several times with distilled water, and these washings were added to the filtrate. The aqueous phase† was then transferred to a volumetric flask and made to volume. *This fraction is referred to in Table II as fraction A.* Its $C^{14}$ content was determined as described below. Lipids were extracted from the residue on the filter paper by a modification of the method described by Sperry *et al.*(8). The residue was transferred, by means of hot

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7. Entenman, C., Lerner, S. R., Chaikoff, I. L., and Dauben, W. G., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 364.

† In several experiments the aqueous phase was thoroughly extracted with petroleum ether and the petroleum ether solution tested for $C_{14}$ content. None was found.

8. Sperry, W. M., Waelsch, H., and Stoyanoff, V. A., *J. Biol. Chem.*, 1940, v135, 281.

TABLE II.

Showing the Extent to Which the Added $C^{14}$ Was Recovered in the Various Fractions in Experiments with Liver Slices.

Each incubation flask contained 350-500 mg of slices, 4.5 cc of Krebs-bicarbonate buffer, and 0.5 cc of a saline solution containing $C^{14}$-labeled glucose. The period of incubation was 3 hours at 37.5°. The contents of 2 or 3 flasks (containing a total of about 1 g of slices) were pooled for analysis. All values refer to percentages of the added $C^{14}$.

| Exp. | $C^{14}O_2$ | $C^{14}$ in Fraction A | Fraction B | | Total $C^{14}$ accounted for |
|---|---|---|---|---|---|
| | | | Fatty acid, $C^{14}$ | Rest, $C^{14}$ | |
| 5 | 4.5 | 94 | .7 | 1.3 | 101 |
| 6 | 3.1 | 97 | .5 | * | 101 |
| 7 | 4.4 | 94 | .7 | * | 99 |
| 8 | 4.2 | 94 | .8 | * | 99 |
| 9 | 5.0 | 89 | * | * | 94 |
| 10 | 5.8 | 88 | 1.6 | * | 95 |
| 11 | 6.0 | 86 | .8 | * | 93 |

* Not determined.

alcohol, to an Erlenmeyer flask and to this was added one cc of 90% KOH. The final alcohol concentration was adjusted to 50 per cent with water (the total volume at this point was about 10 cc), and the mixture was heated on a steam bath for several hours. *This alkaline hydrolysate is referred to as fraction B.* The alcohol was first allowed to evaporate, and enough 95% ethyl alcohol was now added to restore the concentration of alcohol in the mixture to 50%. The unsaponifiable material was removed by 3 extractions (each time with about 75 cc) of petroleum ether. The combined petroleum ether extracts were thoroughly washed, first with 2N KOH and then with distilled water after the manner of Sperry *et al.*(8). These washings were returned to fraction B.

The petroleum ether extract containing the unsaponifiable material was analysed for its $C^{14}$ activity. The $C^{14}$ counts recovered in this fraction were negligible under the conditions studied here. Fraction B was heated on a steam bath to free it of alcohol and then was made acid to bromcresol-green and the acidified mixture allowed to stand for at least one hour at 4° to permit the fatty acids to form aggregates. The precipitated fatty acids were separated by filtering the mixture through No. I Whatman paper. The filtrate was found to contain $C^{14}$ (referred to as rest $C^{14}$ in Table II) which was not extractable with petroleum ether. The fatty acids were dissolved with acetone and the filter paper was boiled with acetone to ensure complete recovery of the fatty acids. The combined acetone solutions were evaporated on a steam bath, and the last traces of this solvent were removed under a stream of $CO_2$. The fatty acids were redissolved in hot petroleum ether and the petroleum ether extract was washed 3 times with distilled water. Flasks equipped with sidearms were used for this purpose. The petroleum ether extract was transferred to a flask containing about 5 g of anhydrous sodium sulfate. The dried petroleum ether solution was made to volume and aliquots were removed for the determination of fatty acid-$C^{14}$.

*Determination of $C^{14}$.* $C^{14}$-containing materials were oxidized to $CO_2$ by the method of Van Slyke and Folch(9). The $CO_2$ evolved was collected as $BaCO_3$ and the $C^{14}$ content of the $BaCO_3$ was determined by the method of Entenman *et al.*(7).

*Results.* In the first 2 experiments (Table I), the conversion of the added glucose-$C^{14}$ to fatty acids was studied. In the next 2 experiments, the incorporation of the $C^{14}$ into $CO_2$ as well as fatty acids was measured. The recoveries of the $C^{14}$ in these fractions are expressed as percentages of $C^{14}$ added to the medium.

*Liver.* From 0.4 to 0.8% of the $C^{14}$ was recovered as fatty acids per g of tissue, and approximately 5% was converted to $CO_2$.

*Kidney.* Only a small fraction of the

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9. Van Slyke, D. D., and Foch, J., *J. Biol. Chem.*, 1940, v136, 509.

added $C^{14}$ was recovered as fatty acid in the experiments with kidney slices.

*Muscle.* Data are presented for both diaphragm and skeletal muscle obtained from the rat's thigh. The thigh muscles were sliced along their longitudinal axes.

The diaphragm sheets oxidized 2.5-3% of the $C^{14}$-glucose to $CO_2$. From 0.02 to 0.2% was recovered as fatty acids. Less of the $C^{14}$ was recovered as fatty acids and $CO_2$ in the experiments with skeletal muscle.

*Recovery of the Glucose-$C^{14}$ in Various Fractions.* Typical results of the $C^{14}$ recoveries in the various fractions separated in the course of experiments carried out with approximately one gram amounts of liver slices are recorded in Table II. At the end of the run, the largest amount of the $C^{14}$ was found in the aqueous phase designated fraction A. Osazone-forming compounds, which we have assumed to be mainly glucose, accounted for about 80 per cent of the $C^{14}$ contained in fraction A.

From 3 to 4.5% of the added $C^{14}$ was converted to $CO_2$. About 2% was found in fraction B, less than half of which was in the form of lipids. The $C^{14}$ recovered in these various fractions accounted for practically all of the glucose-$C^{14}$ added to the medium.

*Comment.* Conclusive evidence that the conversion of glucose to fatty acids can occur in tissues other than the liver was provided in an earlier report from this laboratory(2). Rats that had been maintained on a high carbohydrate diet for 2 or more weeks were deprived of their livers and gastrointestinal tracts by evisceration and were then injected with $C^{14}$-labeled glucose. Palmitic acid containing $C^{14}$ was isolated from these eviscerated preparations. The conclusion based on the eviscerated animal is fully supported by the demonstration that kidney slices and diaphragm convert glucose to fatty acids. An indication of the relative significance of lipogenesis in glucose utilization by isolated liver and diaphragm, can be obtained by comparing for each, the recovery of $C^{14}$ as $CO_2$ with recovery as fatty acids. In the case of the animal used in experiment 4, the ratios were 6 and 18, respectively. Since fatty acids are degraded more rapidly in the liver than in extrahepatic tissues(10), these ratios suggest that fatty acid formation plays a more important role in glucose utilization per gram of liver than per gram of muscle.

*Summary.* 1. The conversion of $C^{14}$-labeled glucose to fatty acids by surviving slices of liver, kidney, and diaphragm is demonstrated.

2. The recoveries of fatty acid-$C^{14}$ in the experiments carried out with liver slices were about 10 times as great as those observed with diaphragm or kidney.

3. The glucose-$C^{14}$ recovered as fatty acid-$C^{14}$ in the experiments with liver slices was 1/6 to 1/10 of that which had been converted to $CO_2$.

4. An incubation flask is described that is suitable for the collection of $CO_2$ evolved in tissue slice experiments from $C^{14}$-labeled compounds.

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10. Goldman, D. S., unpublished observations.

Received January 11, 1950. P.S.E.B.M., 1950, v73.

# Experimental Renal Hypertension in the Dog Following Renal Ischemia.[*] (17680)

EDWARD WM. HAWTHORNE.[†] (Introduced by G. E. Wakerlin.)

*From the Department of Physiology, College of Medicine, University of Illinois, Chicago.*

Experimental renal hypertension can be produced with regularity in the dog by constricting the renal arteries(1-3). Wakerlin and associates have succeeded in producing experimental renal hypertension in 100% of dogs by using a standardized technique of renal artery constriction(4). The present research took advantage of this accuracy in producing experimental hypertension in the dog, by using it as a tool in studying the effects of varying degrees of renal damage on subsequent experimental hypertension production in the same animal.

*Methods.* Nineteen experimental and 10 control dogs were used varying in age, weight, and sex. The experimental dogs were divided into 4 groups and observed for a control period of from one to 2 months. Three groups of 5 dogs each received respectively 2, 4, and 5 hours simultaneous bilateral renal artery occlusion following the control period and the fourth group of 4 dogs received 6 hours of occlusion. A recovery period of one month was allowed each dog after renal artery occlusion. Following this period the dogs were subjected to bilateral renal artery constriction (3-week interval between constrictions) according to the standardized technique referred to above. Mean femoral arterial pressure, blood urea nitrogen (BUN), phenolsulfonphthalein excretion (PSP)(5), and urinalysis changes were observed at semiweekly or more frequent intervals.

*Results.* Ten control dogs, receiving bilateral renal artery constriction without preliminary bilateral renal artery occlusion, all showed a rise in mean femoral arterial pressure averaging 43 mm of mercury for the period of observation which was 4 or more months following bilateral renal artery constriction. The mean arterial pressure change of a typical dog in this group is shown in Fig. 1. The changes in the mean femoral arterial pressure of the dogs which survived the renal damage induced by bilateral renal artery occlusion and subsequent bilateral renal artery constriction demonstrated 2 major patterns which were quite unlike the control group. Seven dogs which developed mild renal damage from 2 and 4 hours of bilateral renal artery occlusion, as evidenced by a modest rise in blood urea nitrogen with slight to no change in phenolsulfonphthalein excretion, evidenced the changes in mean arterial pressure shown in Fig. 2, namely a sharp rise in the mean arterial pressure in the first month following the renal artery constriction. The pressure was seen to fall toward normal in the third month following bilateral renal artery constriction and in some of the dogs the pressure returned almost to the normotensive control level. Four dogs survived severe renal damage, produced in 3 of the dogs by 4 hours and in one by 6 hours of bilateral renal artery occlusion. In these 4 dogs very slight or no rise in pressure was seen for a period of 3 or more months following bilateral renal artery constriction. Two of the dogs were sacrificed at the end of 3 months and 2 of the dogs were observed for a longer period. It will be noted from Fig. 3, which represents the typical changes in mean arterial pressure

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[*] This work was aided by grants from the U. S. Navy, the John and Mary Markle Foundation, the Life Insurance Medical Research Fund, and the Graduate College Research Board of the University of Illinois.

[†] Life Insurance Medical Research Fellow.

1. Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., Munoz, J. M., and Taquini, A. C., Renal Hypertension (translated by L. Dexter), Charles C. Thomas Pub., Springfield, Illinois, 1946.

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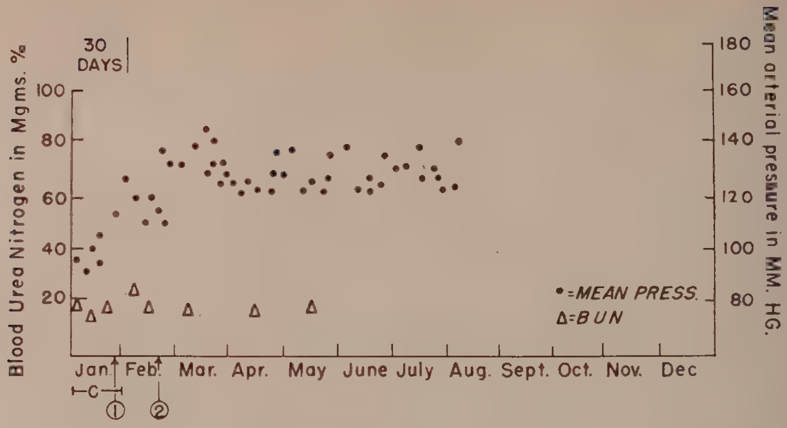

FIG. 1.

A typical control dog showing the changes in the mean arterial pressure (●) following bilateral renal artery constriction [$\overset{\uparrow}{(1)}$, $\overset{\uparrow}{(2)}$]. A rise in pressure was seen in all control dogs. (△) Blood urea nitrogen. ←C→ = Control period.

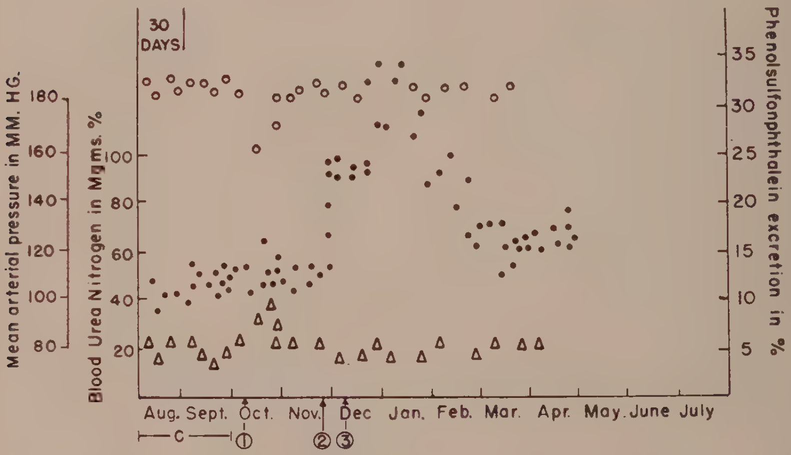

FIG. 2.

Showing the changes in Bun (blood urea nitrogen) concentration (△), PSP (phenolsulfonphthalein) excretion (○), and mean arterial pressure (●), following bilateral renal artery occlusion for two hours $\overset{\uparrow}{(1)}$, and following bilateral renal artery constriction [$\overset{\uparrow}{(2)}$, $\overset{\uparrow}{(3)}$]. ←C→ = Control period.

for the dogs of this group that following bilateral renal artery constriction, the mean arterial pressure remained normotensive for 5½ months and then rose during a subsequent observation period of 2 months.

The other dog became hypertensive after a 3 months' normotensive period following bilateral renal artery constriction. The abrupt rises in pressure seen in these two dogs were not associated with a change in the blood urea nitrogen concentration.

*Discussion.* The results thus far suggest that there is an effect of preliminary renal damage resulting from bilateral renal artery occlusion on experimental renal hypertension production in the dog. No attempt is made at

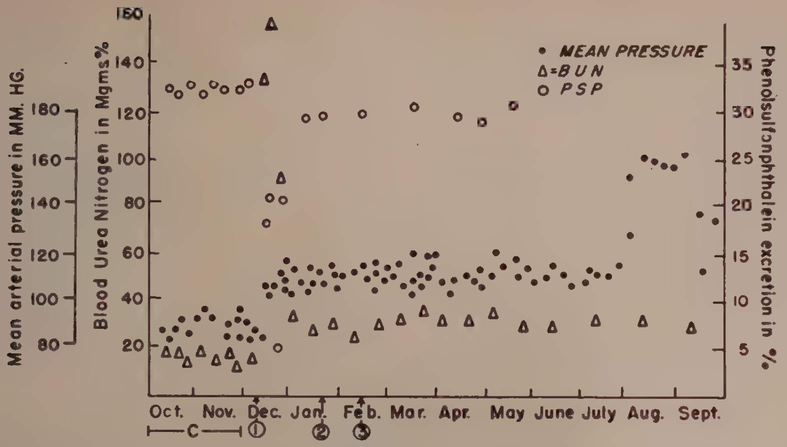

FIG. 3.

Showing the changes in Bun (blood urea nitrogen) concentration (△), PSP (phenolsulfonphthalein) excretion (○), and mean arterial pressure (●), following bilateral renal artery occlusion for four hours $\overset{\uparrow}{(1)}$, and following bilateral renal artery constriction [$\overset{\uparrow}{(2)}$, $\overset{\uparrow}{(3)}$]. ←C→ = Control period.

this time to explain the mechanisms involved (6-9) for it is felt that an expansion of the series of dogs surviving severe renal damage is needed and more detailed information on changes in renal function, pathophysiology and histochemistry is indicated.

*Summary.* The effect of preliminary renal ischemia produced by bilateral renal artery occlusion for 2, 4, 5 and 6 hours on subsequent experimental renal hypertension production on 19 dogs has been studied. In general the results suggest that previous renal damage of both a mild and severe degree has an effect on experimental renal hypertension. Seven dogs receiving mild renal injury showed a very high rise in mean arterial pressure in the first month following bilateral renal artery constriction, and a subsequent fall in pressure to near normotensive levels. The 4 dogs that survived more severe renal damage showed slight to no rise in mean arterial pressure during the first 3 or more months following bilateral renal artery constriction. The dogs of this group which were observed for more than three months showed an abrupt rise in mean arterial pressure, one 3 months and the other 5½ months after bilateral renal artery constriction.

I should like to thank Dr. George E. Wakerlin, who suggested this problem, for his guidance.

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# Effects of Desoxy- and Methoxypyridoxine in the Mouse.[*] (17681)

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A powerful anti-pyridoxine activity of desoxypyridoxine and methoxypyridoxine was observed by Ott(1,2) in the chick. The effects of these compounds in the rat were studied by Porter and co-workers(3). These workers concluded that desoxypyridoxine interferes with some phase of tryptophan metabolism. They also found that, though methoxypyridoxine may act similarly, there was some cleavage of this substance *in vivo* to pyridoxine. The finding reported from this laboratory(4) that the vitamin $B_6$ deficient mouse, in contrast to the rat, does not develop a typical set of symptoms has been confirmed by others(5,6). The observations of Ott suggested to us the possibility of producing a more severe deficiency in the mouse by means of desoxy- and methoxypyridoxine.

*Experimental.* (a) *The effect of desoxypyridoxine and methoxypyridoxine in vitamin $B_6$ deficient mice.* In this study, 3 strains[†] of albino mice were used, namely, Swiss-Webster, Rockland, and our own strain. The animals were placed at weaning on a vitamin $B_6$-free diet (diet A) of the following composition: Casein (Labco), 25; sucrose, 53; salts(7), 5; roughage material (Ruffex) 2; hydrogenated vegetable fat (Crisco or Primex), 10; lard, 5. This diet was supplemented with the following vitamins added per kg diet: Thiamine, 10 mg; riboflavin, 10 mg; calcium pantothenate, 100 mg; choline, 1.5

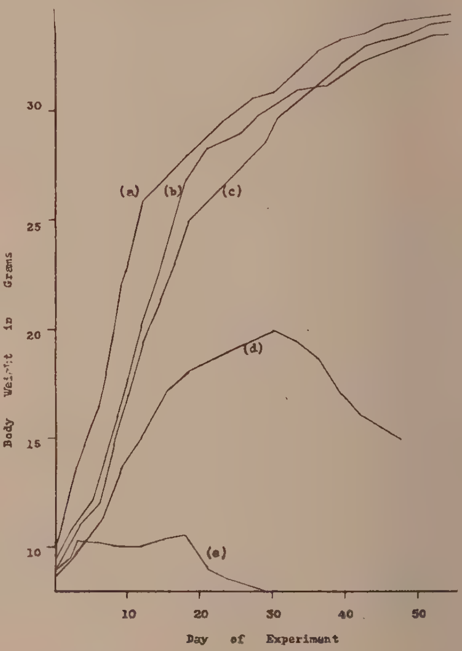

Fig. 1.

Growth curves showing the effects of desoxypyridoxine (DP) and methoxypyridoxine (MP) in vit. $B_6$ deficient mice.

(a) Growth curve of mice receiving the deficient diet (diet A) supplemented with 20 mg MP per kg diet; (b) growth curve of mice receiving diet A and a subcutaneous injection of 50 $\gamma$ MP daily; (c) growth curve of mice receiving diet A supplemented with 20 mg DP and 20 mg MP per kg diet; (d) growth curve of mice receiving diet A, and, (e) growth curve of mice receiving diet A supplemented with 20 mg DP per kg diet.

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[*] This investigation was aided in part by grants from the Committee on Scientific Research of the American Medical Association and the Division of Research Grants and Fellowships of the U. S. Public Health Service. A preliminary report of the present paper has already appeared (American Chemical Society Abstracts, New York Meeting, September, 1947).

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[‡] A mixture of 90% $\beta$- and 10% $\alpha$-carotene was used.

TABLE I.
Effects of Desoxypyridoxine (DP) and Methoxypyridoxine (MP) on Vit. $B_6$ Deficient Mice.

| Diet | No. of mice | Time of appearance of the acrodynia (avg in days) | Survival time (avg in days) |
|---|---|---|---|
| Control | 12 | — | 40 |
| Control + DP | 24 | 23 | 32 |
| Control + MP | 11 | — | * |

* All the mice in this group were normal. Their avg body wt on 60th day of experiment was 34.6 g.

g; $\beta$ carotene‡ 20 mg; $\alpha$ tocopherol, 40 mg; vitamin D (Drisdol) 2500 I.U. This group served as controls. Two other groups received the basal diet supplemented with 20 mg desoxypyridoxine§ and 20 mg methoxypyridoxine,‖ respectively, per kg diet. The results of these experiments are shown in Fig. 1 and Table I.

In Fig. 1 are shown the growth curves of mice of the 3 groups which may be considered typical of the majority of mice studied. Also included is the growth curve of 5 mice which received both analogues at the same level in the deficient diet. Finally, the growth curve of 6 mice given methoxypyridoxine subcutaneously (50 $\gamma$/day) is also included. The anti-vitamin effect of desoxypyridoxine manifests itself both by its effect on growth and its ability to produce the typical vitamin $B_6$ deficiency syndrome (Table I). On the other hand, the methoxy analogue displays pyridoxine activity as indicated by its ability to stimulate growth and counteract the anti-vitamin action of desoxypyridoxine.

(b) *The effect of the protein level in the diet, and the effect of supplementing the diet with methionine, on the deficiency symptoms induced by desoxypyridoxine.* The same experimental procedure was used here as in (a) except that the casein level was varied from 15 to 60% at the expense of sucrose. One group of mice was given the 25% casein diet supplemented with DL-methionine to bring the total methionine content up to the amount present in a 60% casein diet (1.05% DL-methionine). In each case, a control group was run without desoxypyridoxine. The results of these experiments are summarized in Table II. They show that the deficiency symptoms do not develop unless the anti-vitamin is included in the diet. They also show that an increase in the protein intake shortens the survival period of vitamin B deficient mice. This is in agreement with the findings of Miller and Baumann(6). It is worthy of note that a supplement of methionine reproduced the effects of the 60% casein diet.

(c) *The inhibition index of desoxypyridoxine in the mouse.* By 'inhibition index' we mean the molar ratio of anti-vitamin to vitamin, where the quantity of anti-vitamin is just capable of counteracting an amount of the vitamin sufficient to cover minimal daily needs. The procedure used in determining the inhibition index was as follows: Weanling mice were placed on the 25% casein vitamin $B_6$-deficient diet, and kept on this ration for a depletion period of 30 days. At the end of this period, the animals were ready for the experiment. Beginning on the 30th day, and daily thereafter, they were injected subcutaneously with solutions containing desoxypyridoxine and pyridoxine in ratios (anti-vitamin/vitamin) of 50, 25, 15, 10, and 5. Preliminary experiments had shown that an injection of 1 $\gamma$ pyridoxine‖ per day was insufficient for maintenance in most cases, whereas a dose of 2 $\gamma$ would cause some growth, and in some cases a fairly substantial increase in weight. Accordingly, the experiments were performed with both these levels of pyridoxine in the ratios of anti-vitamin/vitamin mentioned above. Table III shows the growth response of the mice to the various ratios of desoxypyridoxine/pyridoxine. It was observed that injections with the higher ratios caused an immediate and sharp decrease in the weights of the animals. When

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§ We are indebted to Dr. Karl Folkers of Merck & Co. for these substances.

‖ Expressed as pyridoxine HCl.

TABLE II.
Data Showing the Effects of the Casein Level in the Diet, and of a Supplement of Methionine, on the Acrodynia Induced by Desoxypyridoxine (DP).

| No. of mice | Level of casein | Appearance of the acrodynia (Avg, days) | Survival time (Avg, days) |
|---|---|---|---|
| 15 | 15% (Control) | — | 49 |
| 16 | 15% + DP | 24 | 31 |
| 12 | 25% (Control) | — | 40 |
| 24 | 25% + DP | 23 | 32 |
| 11 | 45% (Control) | — | 35 |
| 21 | 45% + DP | 24 | 31 |
| 12 | 60% (Control) | — | 30 |
| 18 | 60% + DP | 18 | 25 |
| 10 | 25% + DP + DL-methionine | 18 | 23 |

TABLE III.
Data Showing the Response of Vitamin $B_6$-Deficient Mice to Pyridoxine and to Various Ratios Desoxypyridoxine/Pyridoxine.

| No. of mice | Ratio D.P./P. | $\gamma$ Pyridoxine per day | Growth response* No. of mice + | — |
|---|---|---|---|---|
| 19 | — | 1 | 5 | 14 |
| 14 | — | 2 | 12 | 2 |
| 7 | 50 | 1 | 0 | 7 |
| 7 | 25 | 1 | 0 | 7 |
| 6 | 15 | 1 | 0 | 6 |
| 5 | 10 | 1 | 0 | 5 |
| 4 | 25 | 2 | 0 | 4 |
| 5 | 15 | 2 | 0 | 5 |
| 11 | 10 | 2 | 2 | 9 |
| 7 | 5 | 2 | 5 | 2 |

* The (+) sign denotes a gain, the (—) sign a loss in weight.

desoxypyridoxine was administered at a ratio of anti-vitamin : vitamin of 10 to mice receiving 2 $\gamma$ pyridoxine per day, they still lost weight, though at a slower rate, and then died. On the other hand, when the ratio was 5, under the same conditions, there was a slight increase in the weights of the animals, and they survived for relatively long periods.

All animals receiving the higher ratios of desoxypyridoxine to pyridoxine developed the typical vitamin $B_6$ deficiency syndrome (except those which succumbed rapidly). Curiously enough, even some of the mice receiving the anti-vitamin at the ratio of 5, mentioned in the preceding paragraph, showed the symptoms, but these later disappeared. Such a ratio of 5, in the case of animals receiving only 1 $\gamma$ pyridoxine per day, was still effective. However, this is not significant, since, as mentioned above, 1 $\gamma$ pyridoxine is not adequate for maintenance.

*Discussion.* Contrary to what has been found in the case of rats, simple deprivation of vitamin $B_6$ apparently does not impose a sufficiently severe strain on the mouse to produce the gross symptoms of the deficiency. The experiments described above show that this syndrome may be produced in mice by the administration of desoxypyridoxine. The animals displayed the typical acrodynia of the paws and snout, ring tail, and the spastic gait. In confirmation of the findings of Miller and Baumann(6), our experiments also show that an increase of the casein level in the diet will shorten the survival period of vitamin $B_6$ deficient mice. It is interesting, however, that this increase had no effect on the symptoms induced by the anti-vitamin except when the casein level was increased to 60%, when some slight effect was observed. Apparently at the level of desoxypyridoxine administered, the inclusion of large amounts of casein had only a slight effect on the acrodynia. This is in contrast to the pronounced effect which the casein level exerts on the skin lesions produced in the vitamin $B_6$ deficient rat not receiving desoxypyridoxine(8). The results obtained with the methionine supplement are not surprising in view of our observations on rats(9) which clearly showed the deleterious effect of sulfur amino acids on the vitamin $B_6$ deficient animal.

In contrast to the anti-vitamin action shown by desoxypyridoxine, methoxypyridoxine exhibits pyridoxine activity in the mouse. Thus, the mouse differs from the chick in its reaction to this analogue. The results of Porter and co-workers(3) are of interest in this connection in that they give evidence for both a vitamin and anti-vitamin action of this compound in rats. Results of experiments now in progress in our laboratory indicate that methoxypyridoxine is not quantitatively cleaved to pyridoxine in the mouse, since injection of an amount equivalent to 2 $\gamma$ pyridoxine (which gives a fairly good growth response in the vitamin $B_6$ deficient mouse (Table III)) is without effect.

*Summary.* (1) Desoxypyridoxine has been shown to exert a powerful antipyridoxine effect in the mouse. With the aid of this substance, typical symptoms of vitamin $B_6$ deficiency (acrodynia) have been produced in this species. (2) A high casein level in the diet was found to have a detrimental effect on the acrodynia thus produced, but not to the same extent as that observed in the case of the acrodynia of vit. $B_6$ deficient rats, not receiving desoxypyridoxine. Methionine, in amounts equivalent to those present in the casein, had a corresponding detrimental effect. (3) The pyridoxine requirement of the mouse was found to be, under the conditions of our experiments, between 1 and 2 $\gamma$ per day. (4) For mice receiving 2 $\gamma$ pyridoxine per day, the inhibition index of desoxypyridoxine was found to be 10. (5) Methoxypyridoxine acts as a pyridoxine precursor in the mouse.

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## Hypoalbuminemia and Renal Lesions in Experimental Hyperglobulinemia.*

(17682)

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Recent observations have stimulated interest in the role of proteins in the pathogenesis of renal disease(1-5). This report is concerned with changes in blood serum and kidneys which occur after the intraperitoneal injection of normal rabbit globulin. When the rabbit antipneumococcic serum† employed was subjected to chemical and electrophoretic analysis, it was found to be a 10% globulin solution with a salt content of 0.9% at pH 7.4 consisting chiefly of gamma and traces of beta globulin preserved in 0.4% phenol and 1:50,000 phenyl mercuric borate.

*Method.* Rabbits weighing 2 to 3 kg were fed pellets, lettuce and water. Fifty to 60 cc of the test material were injected intraperi-

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* Work done under a grant from the Joseph and Helen Yeamans Levy Foundation established in memory of their daughter, Miriam Levy Finn.

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† Through the courtesy of the Lederle Laboratories Division of the American Cyanamid Corporation.

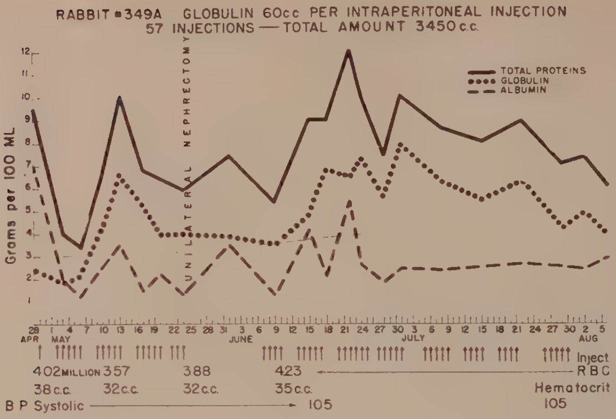

FIG. 1.

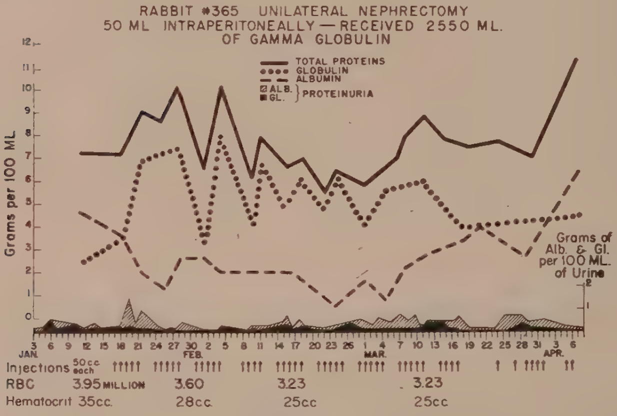

FIG. 2.

The blocked areas represent levels of proteinuria. Urine globulin levels are in black; urine albumin levels are hatched.

toneally daily with interruption over Saturday and Sunday. Nine animals were given globulin and one Ringer's solution. Total daily urine output was recorded 5 days weekly; week-end urine was collected Monday. Unilateral nephrectomy was performed in 4 instances. Four to 5 cc of blood were withdrawn from an ear or by cardiac puncture twice weekly. Serum protein, albumin, globulin(6), blood urea N, cholesterol, hematocrit and erythrocyte counts were estimated, in addition to serum sodium, potassium and cephalin flocculation. The urine total protein, albumin, globulin, urea and chlorides were determined 5 days weekly.

*Results.* There was a rise in the serum globulin and a fall in the serum albumin in all 9 animals receiving the globulin intraperitoneally for 4 or more days. This was usually sustained for the period during which injections were continued. While there was often a slight rise in the total protein, it rarely approached the marked ascent observed in the globulin level. (Fig. 1,2). As may be seen in Table I, the maximum globulin level was 8.15 g per 100 cc rising from an initial level of 2.54 g per 100 cc. The minimum albumin level was 0.64 g per 100 cc. The mean maxi-

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6. Looney, J. M., and Walsh, A., *J. Biol. Chem.*, 1939, v130, 635.

TABLE I.

| Globulin received, cc | Initial total protein, g/100 cc | Max. total protein, g/100 cc | Initial globulin, g/100 cc | Max. globulin, g/100 cc | Initial albumin, g/100 cc | Min. albumin, g/100 cc |
|---|---|---|---|---|---|---|
| 450 | 6.40 | 7.62 | 1.62 | 4.75 | 4.78 | 1.75 |
| 2800* | 5.70 | 10.40 | 1.37 | 5.34 | 4.33 | 1.56 |
| 400 | 5.82 | 6.90 | 1.98 | 4.85 | 3.84 | 2.05 |
| 550 | 5.96 | 7.88 | 2,08 | 5.84 | 3.88 | 2.04 |
| 600 | 8.40 | 8.40 | 1.97 | 5.43 | 6.42 | 1.56 |
| 2850* | 7.20 | 10.16 | 2.54 | 8.15 | 4.65 | 0.64 |
| 350 | 7.50 | 5.86 | 1.56 | 4.20 | 5.95 | 1.66 |
| 1100† | 8.46 | 9.58 | 2.72 | 6.23 | 5.73 | 2.10 |
| 3450* | 9.48 | 12.00 | 2.40 | 7.81 | 7.07 | 1.83 |
| 950 | 8.12 | 11.60 | 3.04 | 6.98 | 5.08 | 2.60 |
| Control Ringer’s sol. 1750 | 5.68 | 6.42 | 1.84 | 1.93 | 3.84 | 3.57 |

* Glomerular and tubular changes.
† Tubular changes only.

mum globulin rose to 2.64 times its original level in the serum, whereas the original albumin level was 2.8 times higher than the mean minimum recorded. In contrast to these findings there was no significant change in the protein levels observed in the control animal which received 27 injections of Ringer’s solution. Blood cholesterol, blood urea N, serum sodium and potassium revealed no persistent abnormalities. The rise in globulin could not be associated with changes in the hematocrit or red blood counts.

*Urine.* The was no constant increase in either albumin or globulin excretion, the average total protein being 0.4 g/100 cc, of which 0.13 g were globulin and 0.27 g albumin. (Fig. 2). Urea content was 0.4 to 2.8%. Chlorides from 0.1 to 1.6%. Daily benzidine tests were constantly negative.

*Glomerular Findings.* Whereas no characteristic morphologic alterations were observed in animals receiving smaller amounts of globulin, all of the 3 rabbits which received globulin in amounts of 2500 cc or more showed a characteristic lesion in many glomeruli (Fig. 3) consisting of a homogenous nodular coagulum of varying size reminiscent of intercapillary glomerulosclerosis (Kimmelstiel-Wilson)(7). In no instance did these nodules give the tinctorial reactions for amyloid when stained with congo red, methyl violet and iodine.

*Tubular Findings.* The cells of the proximal convoluted tubules were laden with droplet material (Fig.(3) which represented tubular reabsorptive activity for protein as revealed by the Gram stain(8).[‡]

*Discussion.* These data may explain the frequent occurrence of hypoalbuminemia in diseases characterized by hyperglobulinemia such as multiple myeloma, kala-azar, malaria and others. Previous experiments by Dick and Leiter(9) with a rabbit plasma globulin were associated with amyloidosis but no correlation between the total amount of globulin administered and such deposits was evident, the amyloid deposits having been attributed to the denatured globulin. The recent production of glomerulonephritis by the intravenous injection of bovine gamma globulin(10) in rabbits was not paralleled in any of the 9 rabbits. That globulin may be concerned with the Kimmelstiel-Wilson lesion is reflected by the findings of others(11,12) who observed hyper-

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7. Kimmelstiel, P., and Wilson, C., *Am. J. Path.*, 1936, v12, 83.

8. Oliver, J., *J. Mt. Sinai Hosp.*, 1948, v15, 175.

[‡] Through the kindness of Professor Jean Oliver, Department of Pathology, Long Island College of Medicine, to whom we are indebted for much guidance.

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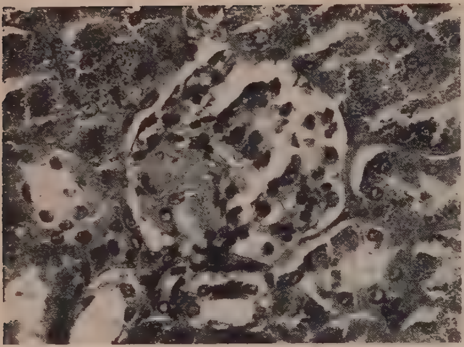

FIG. 3.
Rabbit No. 349 A.
Note the eccentric globoid blomerular lesion. The cells of the proximal convoluted tubules are laden with droplet material of protein origin.

beta-globulinemia and hypoalbuminemia in alloxan treated rabbits and in diabetic humans. This supports the statement of Blackman *et al.*, that hyaline coagula seen in the kidney, not identifiable as fibrin consist chiefly of precipitated globulins.(13)

*Summary.* (1) Homologous gamma globulin injected intraperitoneally elevated the level of serum globulin and depressed the albumin without inducing significant albuminuria.

(2) Characteristic glomerular and tubular changes occurred in the animals in which protracted hyperglobulinemia was produced.

(3) Serum protein levels reverted to normal when injections were discontinued.

(4) Glomerulonephritis was not present.

(5) The resemblance of the glomerular lesions to the nodular lesion of intercapillary glomerulosclerosis is noteworthy.

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13. Blackman, S. S., Jr., Barker, W. H., Buell, M. V., and Davis, B. D., *J. Clin. Invest.*, 1944, v23, 163.

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# Effect of Single Inocula of *Endamoeba histolytica* Trophozoites on Guinea Pigs.*† 17683)

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Guinea pigs are very susceptible to infections with *E. histolytica* of human origin. Carrera and Faust(1) reported that 33 out of 34 guinea pigs inoculated into the terminal ileum with doses of 200,000 or more amebae developed typical amebic lesions. Taylor and coworkers(2) found an infectivity rate of 58%, which could be increased up to 100% with certain dietary modifications. In order to study further certain aspects of this host-parasite relationship the following experiments were carried out.

*Materials and Methods.* A total of 84 guinea pigs of both sexes and of approximately the same weight and age were kept in separate cages through the experiments and were fed mixed Rabbit and Guinea Pig Diet. The operative procedure was the same as that described by Carrera and Faust(1) except that the inoculum was injected directly into the cecal pouch. Aseptic technic was used throughout the operation. A clone which had been isolated some months previously from our strain no. 22 was maintained, together with the bacteria associated with the parent strain, by serial passage every 48 hours in Balamuth's medium(3). The trophozoites used for inoculation were collected by pooling the content of several flasks which had been

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* Supported by a grant from the Division of Research Grants and Fellowships, National Institutes of Health, Bethesda, Md.

† Thanks are extended to Dr. Huldah Bancroft, Professor of Biostatistics in our Department, for checking our statistical interpretations.

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2. Taylor, D. J., Greenberg, J., and Coatney, G. R., *J. Parasit.*, 1949, v35, 24.

3. Balamuth, W., *Am. J. Clin. Path.*, 1946, v16, 380.

TABLE I.
Effect of Graded Numbers of *E. histolytica* on Production of Intestinal Lesions in Guinea Pigs.

| | | | Results at necropsy within 20 days from time of inoculation | | | | |
|---|---|---|---|---|---|---|---|
| | | | No. of animals in group | | Accumulative total | | |
| Group No. | No. of animals | No. of amebae in inoculum | Without lesions | With lesions | Without lesions | With lesions | % infectivity |
| I | 11 | 200,000 destroyed by freezing | 11 | 0 | 46 | 0 | 0 |
| II | 7 | 100 | 7 | 0 | 35 | 0 | 0 |
| III | 8 | 500 | 8 | 0 | 28 | 0 | 0 |
| IV | 8 | 1000 | 7 | 1 | 20 | 1 | 5 |
| V | 8 | 5000 | 4 | 4 | 13 | 5 | 28 |
| VI | 9 | 10,000 | 5 | 4 | 9 | 9 | 50 |
| VII | 13 | 100,000 | 2 | 11 | 4 | 20 | 83 |
| VIII | 9 | 500,000 | 1 | 8 | 2 | 28 | 93 |
| IX | 7 | 2,500,000 | 0 | 7 | 1 | 35 | 97 |
| X | 4 | 5,000,000 | 1 | 3 | 1 | 38 | 97 |

Fifty per cent infective dosage = 10,000 amebae.

inoculated with a known number of trophozoites 48 hours before. The pooled material was then centrifuged for approximately 5 minutes at a speed of 1800 rpm and the supernate removed. Centrifugation at the above speed up to 15 minutes has been found to produce no harmful effect on the viability and multiplication of the amebae. The number of trophozoites per ml of medium was calculated after counting a representative sample in a hemocytometer chamber. Dilutions with the supernate were then made to obtain the desired number of amebae per ml. The volume of inoculum was always the same; *viz.,* 1 ml for the guinea pigs that received less than 100,000 amebae each, and 2 ml for those that received 100,000 or more. The animals were weighed on the day of inoculation and again every 5 days up to the time of necropsy 20 days later. They were sacrificed by exsanguination under nembutal anesthesia. Immediately thereafter the body weight was recorded, the abdominal cavity was opened and the intestines were carefully examined for any gross evidence of ulceration. A sample of the cecum was promptly fixed in Bouin's fluid for histologic examination. Fecal films made from the cecal content were then examined under the microscope for the presence of *E. histolytica.* When guinea pigs were found dead they were inspected for gross evidence of intestinal ulceration and the cecum was examined for the presence of amebae. Whenever the guinea pig had died within a few hours, histologic examination of the cecum was carried out as above.

A few animals that died as a result of anesthesia or of other causes unrelated to amebiasis are not reported. Eighty-four guinea pigs included in this series belonged to 10 groups. Seven each received 100 amebae (Group II), 8 received 500 (Group III), 8 received 1,000 (Group IV), 8 received 5,000 (Group V), 9 received 10,000 (Group VI), 13 received 100,000 (Group VII), 9 received 500,000 (Group VIII), 7 received 2,500,000 (Group IX) and 4 received 5,000,000 (Group X). It was planned that if none of the animals of Groups II and III became infected they were to serve as controls.

In order to test whether the lesions which might be produced were derived from concentrates of bacteria and/or from products of decomposed amebae, about 50 ml of medium containing 100,000 active amebae per ml were dispensed into 2 tubes. The contents of one tube were immersed in a bath of alcohol and dry ice which had attained a temperature of approximately –70°C and were rapidly twirled to insure quick freezing. After 5 minutes the tube was placed in the incubator at 37°C and thawed. Samples from this tube were then examined by direct micro-

TABLE II.
Effect of Graded Numbers of *E. histolytica* on the Survival of Guinea Pigs.

| | | Results within 20 days from time of inoculation | | | | | |
|---|---|---|---|---|---|---|---|
| | | No. of animals in group | | Accumulative total | | | |
| Group No. | No. of amebae in the inoculum | Alive | Dead | Alive | Dead | % mortality | Avg from inoc. to death, in days |
| I | 200,000 destroyed by freezing | 11 | 0 | 53 | 0 | 0 | — |
| II | 100 | 7 | 0 | 42 | 0 | 0 | — |
| III | 500 | 8 | 0 | 35 | 0 | 0 | — |
| IV | 1000 | 8 | 0 | 27 | 0 | 0 | — |
| V | 5000 | 4 | 4 | 19 | 4 | 17 | 10.7 |
| VI | 10,000 | 5 | 4 | 15 | 8 | 34 | 10.7 |
| VII | 100,000 | 5 | 8 | 10 | 16 | 61 | 10.4 |
| VIII | 500,000 | 3 | 6 | 5 | 22 | 81 | 9.2 |
| IX | 2,500,000 | 1 | 6 | 2 | 28 | 93 | 6.5 |
| X | 5,000,000 | 1 | 3 | 1 | 31 | 97 | 8.0 |

$$\text{Proportional distance} = \frac{50 - 34}{61 - 34} = 0.59.$$

$$LD_{50} = 10{,}000 + (90{,}000 \times 0.59) = 63{,}000 \text{ amebae.}$$

scopic examination and by culture and found positive for bacteria and negative for living amebae. Two ml of the medium thus treated were then inoculated intracecally into each of 11 guinea pigs (Group I). One ml of the contents of the tube that had not been immersed in the freezing bath was inoculated into each of 4 guinea pigs, which became infected.

*Results. Relationship between size of inoculum and infection.* As indicated in Table I, twenty days after inoculation, there was no evidence of infection in the controls that received the residue from 200,000 amebae treated by rapid freezing (Group I). In the groups inoculated with living amebae the number of infected animals was proportionately greater as the size of the inoculum was increased.

The $ED_{50}$ for guinea pigs under these experimental conditions was 10,000 amebae. Animals were regarded as infected only when they presented typical gross or microscopic lesions in which trophozoites of *E. histolytica* were seen; and in every case in which a diagnosis of amebic infection was made by gross examination of the lesions and by finding amebae in the cecal content microscopic examination revealed typical ulcers with amebae. *Vice versa*, in every case in which no lesions were observed after a careful gross examination and no amebae were found in the cecal content, no lesions were observed by microscopic examination. The amebae observed in the cecal content of animals found infected at necropsy 20 days after infection were often few in number, rounded up and moribund, while those observed in guinea pigs that died as a result of the infection several days after inoculation were abundant, very active, and not uncommonly were seen with ingested red blood cells. In the great majority of animals the infection overwhelmed the natural resistance of the guinea pigs and caused their death. A perusal of Table II shows that the number of animals which died as a result of the infection was proportionately greater as the size of the inoculum was increased. The $LD_{50}$ was found to fall between 10,000 and 100,000 amebae (Groups VI and VII). By interpolation of the proportional distance it was found to be approximately 63,000. Death occurred in all cases between 5 and 17 days after inoculation. On the average, death occurred 9.3 days after inoculation and the greatest number of guinea pigs died on the eighth day. In general, the size of the inoculum was found to have an inverse relationship to the average time between inoculation and death. While not significant from one size of

TABLE III.

Effect of Body Weight of Guinea Pigs on Their Susceptibility to Infection with *E. histolytica*.

| Group No. | No. of amebae in inoculum | Animals that became infected | | Animals that resisted infection | |
|---|---|---|---|---|---|
| | | No. | Mean wt | No. | Mean wt |
| IV | 1000 | 1 | 195 | 7 | 256 |
| V | 5000 | 4 | 205 | 4 | 313 |
| VI | 10,000 | 4 | 230 | 5 | 244 |
| VII | 100,000 | 11 | 193 | 2 | 163 |
| VIII | 500,000 | 8 | 219 | 1 | 265 |
| IX | 2,500,000 | 7 | 204 | 0 | — |
| X | 5,000,000 | 3 | 183 | 1 | 190 |
| Totals | | 38 | | 20 | |
| Avg | | | 205 | | 252 |

inoculum to another, the average time from infection to death of the guinea pigs that received 5,000 amebae each (Group V) and those that received 2,500,000 amebae each (Group IX) was found to be significant. The values obtained using the *t* test(4) are:

$$t = 3.94;\ 0.01 > p > 0.001.$$

The apparent rise in the average time for the group receiving an inoculum of 5,000,000 amebae may be due to the fact that only 4 animals were inoculated.

*The relationship between body weight and infection.* Although in the course of these experiments guinea pigs of approximately the same size and age were chosen, some observations were possible on the relationship between body weight and infection. First, infection with *E. histolytica* produced a rapid loss of weight of the experimental animals. The mean weight at the time of inoculation of the 31 guinea pigs that died was 202 g, while at the time of death, on the average 9.3 days later, it was 151 g. Also, the weight at time of inoculation seemed to have an inverse correlation with the degree of susceptibility of the guinea pigs to infection with *E. histolytica*. Table III shows that in every group but one (Group VII) the average weight at the time of inoculation of the guinea pigs which became infected, was considerably lower than that of the guinea pigs which resisted the infection. On the average, the mean weight of 38 guinea pigs which later became infected, was 205 g at the time of inoculation, while that of the 20 guinea pigs that did not become infected was 252 g.

The difference in weight at the time of inoculation of the guinea pigs which later became infected and those that were not found infected (Groups IV through X) was tested by comparing the difference in weight to the standard error of the difference. The values obtained are:

$$\frac{\text{actual diff.}}{\text{S.E. diff.}} = \frac{47}{15} = 3.15$$

For this value P = 0.002. The difference is therefore regarded as significant.

*Histopathologic studies.* These were limited to the cecum, because as previously reported(1) the cecum is always involved whenever amebic lesions occur in the guinea pig. Five representative sections per animal were studied. Prompt fixation of the tissues in Bouin's fluid, and staining of the sections with hematoxylin and eosin allowed the amebae to be readily distinguished. The architecture of the amebic lesions in the present series, was found to be similar in all repects to that previously reported(1) *viz.*, extensive superficial areas of invasion of the mucosal epithelium, and narrower, deeper lesions with an occasional perforation of the whole thickness of the intestinal wall. Whenever the amebae were found in association with large bacterial colonies, there was intense local cellular exudation and necrosis. But at times amebae were seen colonizing the subepithelial tissues, without evidence of much host-tissue reaction and without apparent concomitant bacterial invasion. The presence of *Balan-*

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4. Fisher, R. A., *Statistical Methods*, 10th Ed., 1948, 122.

*tidium caviae* in some of the infected animals as well as in some of the controls did not appreciably alter the character of the normal mucosa. Whenever this ciliate was seen in the neighborhood of an amebic lesion, it did not appear to be in the advancing border of the lesion but was situated in the necrotic menstruum filling the crater of the ulcer. In rare instances it was seen in the subepithelial spaces. In the control animals and those reported as negative no ulcers of any kind were observed in the cecal mucosa; on the other hand, all the animals found to be positive by gross examination and by direct smear of the cecal feces, were found on histologic examination to have typical amebic lesions.

*Summary*. Guinea pigs were inoculated intracecally with graded single doses of from 100 to 5,000,000 amebae. None of the animals that were given less than 1,000 amebae developed infection. The 50% infective dose under the experimental conditions was 10,000 amebae. The mean weight of the guinea pigs that later became infected was significantly smaller than that of those which resisted infection. Thirty-one out of 50 guinea pigs that received single inocula of from 5,000 to 5,000,000 amebae died as a result of the infection. The $LD_{50}$ under the experimental conditions was 63,000 amebae. Death occurred in all cases between 5 and 17 days after inoculation, on the average 9.3 days after inoculation, and the greatest number of guinea pigs died on the eighth day. In general, the size of the inoculum was found to have an inverse relationship to the average time between inoculation and death. Histopathologic studies confirmed necropsy examination. All animals found to be positive by gross examination and by direct smears of the cecal content were found to have typical amebic lesions, while in none of the controls and of those reported as negative were ulcers of any kind observed by histologic sections.

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## Relationship Between Physiological Disposition and Adrenolytic Action of 2-(N-p′-tolyl-N-m′hydroxy-phenyl) - aminomethyl) - imidazoline hydrochloride (C-7337).* (17684)

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The relationship of physiological disposition to pharmacological action of adrenolytic agents has not been widely studied because of the lack of sensitive chemical tests for those agents which have been most extensively used up to the present time. The discovery of 2-(N-p′-tolyl-N-m′hydroxyphenyl-aminomethyl)-imidazoline hydrochloride (C-7337) has for the first time made such investigations possible since this compound may readily be determined in biological fluids and is a potent adrenolytic agent of low toxicity(1-5).

*Chemical methods.* A number of possible reactions involving the free phenolic group of C-7337 was investigated. The most satisfactory was found to be a modification of the 4-amino-antipyrine color reaction(6). The

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* The authors wish to thank Dr. F. F. Yonkman of the Ciba Pharmaceutical Products, Inc., for the generous supply of C-7337.

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3. Grimson, K. S., Chittum, J. R., and Longino, F. N., *Fed. Proc.*, 1949, v8, 61.

4. Meier, R., Yonkman, F. F., Craver, B. N., and Gross, F., Proc. Soc. Exp. Biol. and Med., 1949, v71, 70.

5. Walker, H. A., Wilson, S., Heymans, C., and Richardson, A. P., *Arch. int. Pharmacodyn.*, 1950, in press.

procedure as finally adopted for plasma, whole blood and urine was as follows: One ml of material to be analyzed was shaken mechanically with 0.5 ml of saturated sodium bicarbonate and 9.0 ml of chloroform for 10 minutes in a glass stoppered centrifuge tube. Sample was centrifuged for 3 minutes at 1000 RPM and the water layer removed by suction. A 7.0 ml aliquot of the chloroform layer was transferred to a test tube and chloroform removed by evaporation from a water bath. Four ml of distilled water were added to the residue and the sample heated for 5 minutes at 90-100°C to insure complete solution. On cooling, 1.0 ml of amino-antipyrine reagent (1.36 g of 4-amino-antipyrine to 1 liter of water) was added and mixed. One-half ml of alkaline ferricyanide solution (8.67 g of potassium ferricyanide and 1.8 ml of C.P. ammonium hydroxide made up to 1 liter with water) was added and the intensity of the resulting pink color was determined with a Beckman Spectrophotometer at 490 m$\mu$. All samples were read between 5 and 10 minutes after final addition of the reagents which was the period of maximum development of color.

Approximately 85% of C-7337 added to water, plasma, and urine in concentrations of 1-100 $\gamma$/ml was recovered. As described, the method was not a suitable one for determining this drug in tissue since recoveries were poor and uncertain. This was especially true in the case of brain.

Preliminary studies indicated that approximately 10% was the maximum amount of C-7337 which could be recovered unchanged from the urine after intravenous administration. This observation led to a search for metabolic products. Attempts to extract material which coupled with amino-antipyrine from urine under acidic or basic conditions were unsuccessful. However, it was found that when urine was first hydrolyzed with 1.0 N hydrochloric acid and then brought back to the pH at which the free C-7337 had been extracted, approximately twice as much material was recovered. The procedure for the determination of "total" drug was as follows: 2.0 ml of urine were heated with 2 ml of 1.0 N HCl for 15 minutes in a boiling water bath. After cooling 4 ml of saturated sodium bicarbonate were added and 1.5 ml of the resulting mixture were shaken with 9 ml of $CHCl_3$ and treated as described for the determination of free C-7337.

*General Plan of the Experiments.* Dogs which had been fasted for 12 hours were secured to an operating table and prepared for intravenous injections and recording of blood pressure and pulse rate. After the response of each animal to control injections of U.S.P. epinephrine, C-7337 was administered and epinephrine responses retested, from time to time, until return to normal. Blood and urine samples were collected at appropriate intervals for determination of drug concentration. For the most part, animals were unanesthetized. However, in the case of some of the experiments in which it was necessary for dogs to be secured to the operating table for long periods of time, it was found necessary to administer morphine subcutaneously in a dosage of approximately 1.0 mg/kg. With the higher doses of drug, in which there were still adrenolytic action at the end of 20 hours, a number of experiments were carried out in which C-7337 was given the evening before test responses to epinephrine were begun.

*Results.* A series of 41 dogs was treated intravenously and orally with varying doses of the drug in order to determine the relationship between absorption, plasma concentration, excretion, and adrenolytic activity. The general kind of results obtained is illustrated by Fig. 1, which summarizes the results obtained following the administration of 15 mg/kg intravenously to a group of 5 dogs. Following this dose of C-7337 epinephrine reversal was produced for a period longer than 18 hours. In spite of this prolonged action, the drug disappeared rapidly from plasma and could not be detected after 1 hour (plasma level below 1 $\gamma$/ml). However, C-7337 continued to be excreted in the urine for as long as 24 hours, a total of 13.8% free C-7337 being recovered within a 24 hour period of observation. Thus it appears likely that C-7337 is extensively localized in tissues and it is this localization which determines the

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6. Emerson, E., *J. Org. Chem.*, 1943, v8, 417.

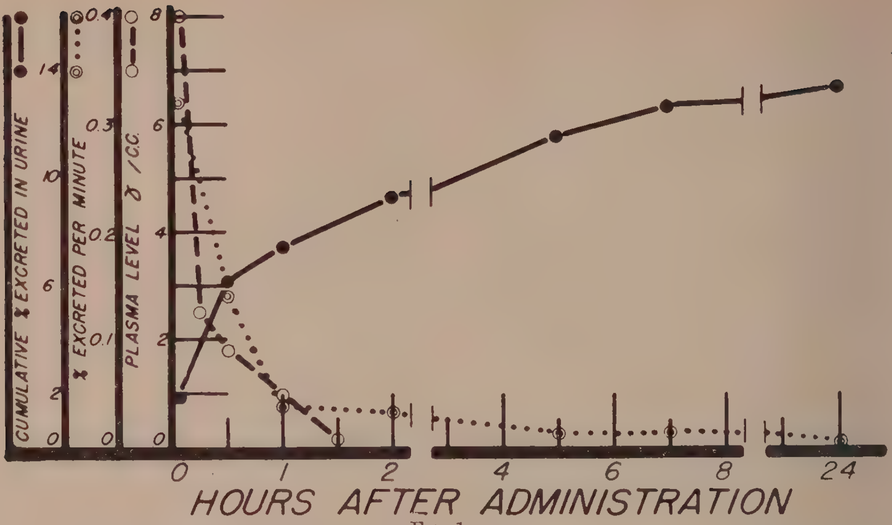

FIG. 1.

Plasma concentration and urinary excretion of C-7337 in terms of "free" drug after the intravenous injection of 15 mg/kg. Mean of 5 experiments.

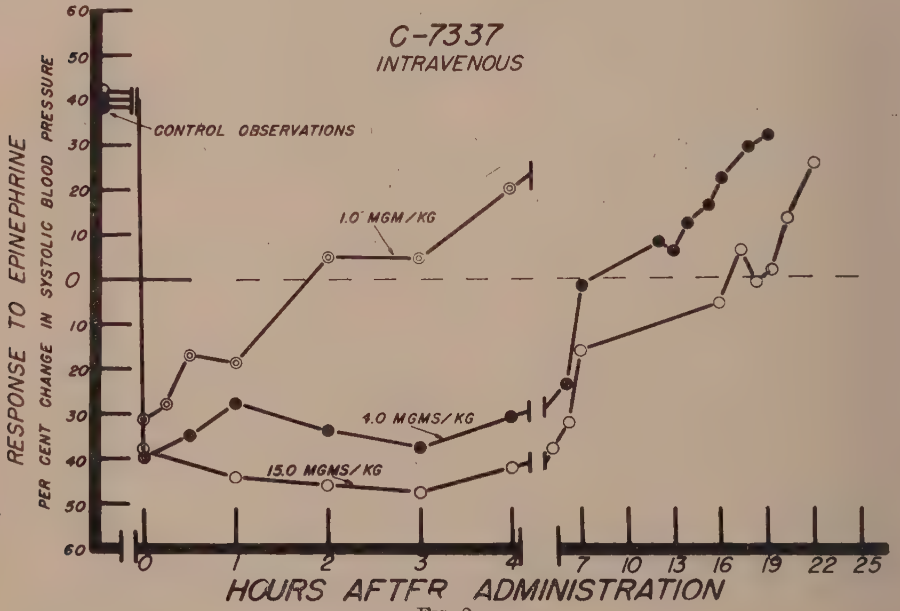

FIG. 2.

The effect of 3 different intravenous doses of C-7337 on the response to the intravenous injection of epinephrine.

degree and duration of adrenolytic activity.

Data which illustrate the relative effectiveness of oral and intravenous administration of C-7337 in blocking the pressor action of epinephrine are summarized by Fig. 2 and 3. It is apparent that increasing the dose of the adrenolytic agent increases the intensity of the response only up to a certain point

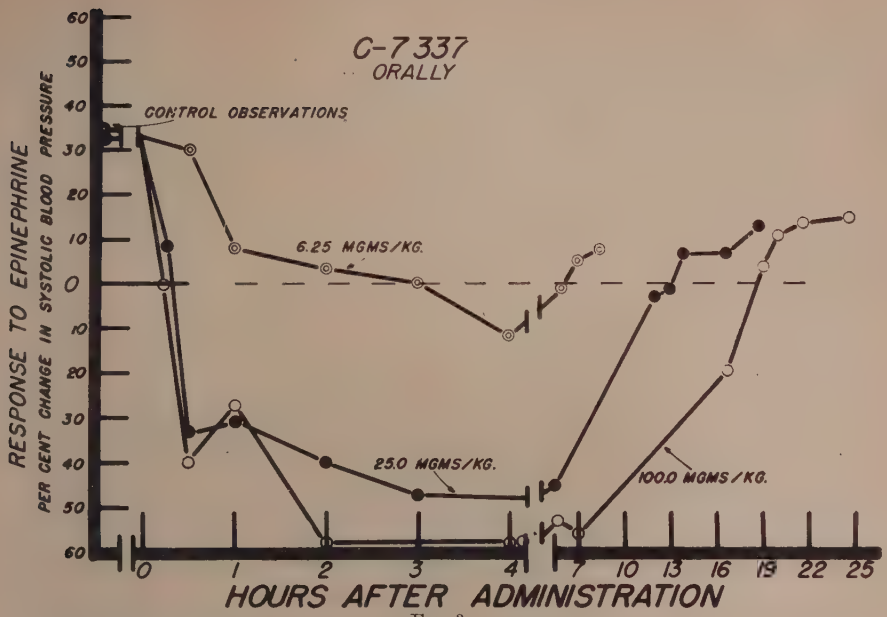

FIG. 3.

The effect of 3 different oral doses of C-7337 on the response to intravenous injection of epinephrine.

beyond which the effect of increasing dosage was manifest only by an increase in the duration of blockade. Comparing the effect of 1.0 mg/kg intravenously to 6.25 mg/kg orally it is also apparent that the injection of the drug causes a more intense blocking action than when the drug is introduced slowly into the circulation as by absorption from oral administration. In terms of maximum degree of action intravenous administration is many times more effective than oral administration. However, if the two routes of administration are compared as to duration of action, this difference is much less marked.

Table I summarizes relationship of adrenolytic activity to plasma levels and urinary excretion of drug. These data show that in terms of both maximum degree and duration of blockade, intravenous administration is approximately 6 times as effective as oral administration. The fact that peak plasma levels with oral administration were observed at 15 or 30 minutes would seem to indicate that absorption from the gastro-intestinal tract is rapid. With doses of 100 mg/kg orally the plasma concentration decreased more slowly from the peak level of 4 $\gamma$/ml than was seen after a single intravenous dose. The data in Table I also demonstrate that complete blockade and reversal of the pressor response of epinephrine may occur without plasma concentrations even reaching a level of 1 $\gamma$/ml. These findings may be explained on the basis of rapid localization within some tissue or body fluid. From the time-plasma concentration curves showing the disappearance of the drug from the blood following a single intravenous dose the apparent volume of distribution was estimated by extrapolating the curves back to zero time. This value also indicated extensive tissue localization.

Despite the fact that the per cent urinary excretion of total drug was generally greater after intravenous injection than after oral administration, a greater percentage of the total drug recovered was conjugated with oral administration (52-65%) than after intravenous injection (31-41%). This suggests that gradual absorption from the gastro-intestinal tract may favor conjugation.

*Discussion.* No definitive information has

TABLE I.
Summary of Data Showing Relationship of Plasma Concentration and Urinary Excretion to Adrenolytic Activity of C-7337.

| Dose, mg/kg | No. of animals | % of dose recovered in urine | | Blockade of epinephrine pressor effect | | Mean peak plasma level, $\gamma$/ml | |
|---|---|---|---|---|---|---|---|
| | | Free | Total | Onset min. | Duration in hr | Conc., $\gamma$/ml | Min. after administration |
| | | | | Intravenously | | | |
| 1. | 7 | 8.5 | 12.4 | <2 | 2 | <1.0 | — |
| 4. | 6 | 8.7 | 12.9 | <2 | 10 | 8.7 | 0–5 |
| 15. | 8 | 13.8 | 23.3 | <2 | 18 | 8.3 | 0–5 |
| | | | | Orally | | | |
| 6.25 | 6 | 2.6 | 5.4 | 120 | 2 | <1.0 | — |
| 25. | 7 | 4.9 | 13.9 | 15 | 12 | 1.2 | 15–30 |
| 100. | 7 | 4.3 | 10.4 | 15 | 18 | 4.0 | 15–30 |

been obtained as to the metabolic fate of C-7337 or the nature of the conjugated material which can be detected in the urine. A small amount of impure hydrolyzed metabolic product has been collected and tested for its pharmacological action. The concentration of the material used for these biological tests was determined in terms of material which coupled with 4-amino-antipyrine. It was found that this metabolic product did possess adrenolytic action, although it was less than ¼th as active as an equal amount of pure C-7337 and was shorter in duration of effect. Since the material isolated was not pure the only conclusion which can be drawn from these experiments is that some of the metabolic products have lost their effectiveness as adrenolytic agents.

*Summary.* 1) A chemical method is described for the determination of C-7337 in plasma and urine which is sensitive to at least a concentration of 2 $\gamma$/ml.

2) In terms of duration of blockade of epinephrine pressor effect, C-7337 is approximately 1/5th as active by mouth as by intravenous injection.

3) Approximately 10% free C-7337 may be recovered in the urine following intravenous injection whereas only one half this amount may be recovered after oral administration.

4) C-7337 disappears rapidly from plasma but continues to be present in the urine for as long as 24 hours. These results indicate that the drug is extensively localized in some tissues of the body.

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## Interference by Glucose in the Quantitative Determination of Uric Acid.* (17685)

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A notable increase in uric acid and glucose excretion was observed in a patient with juvenile rheumatoid arthritis (Still's disease) while receiving cortisone and also when given ACTH. The parallelism between observed values of uric acid and glucose was impressive and suggested the need to check the effect of glucose on the values obtained for uric acid by the method used. The method for the determination of uric acid was that of Buchanan, Block and Christman(1) in which uric

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* This work is part of a study supported by a grant from the Masonic Foundation for Medical Research and Human Welfare.

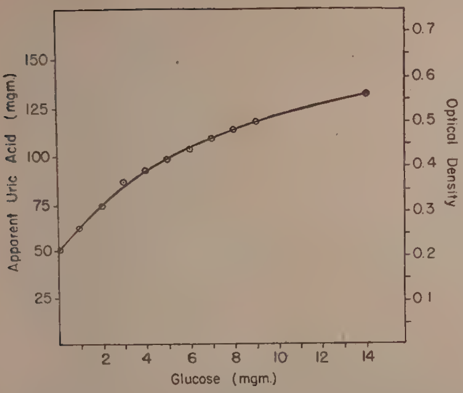

FIG. 1.

Effect of increasing glucose concentration on a constant amount of uric acid. 50 μg of uric acid in 50 ml of water. Read at 660 μ in a Coleman junior spectrophotometer.

acid is determined as the difference in values obtained before and after digestion with the enzyme uricase. Application of this method to solutions of uric acid, of glucose, and of mixtures of uric acid and glucose gave the following results:

a. Color formation from given amounts of glucose and of uric acid in mixtures of the two was far greater than that found when either was used alone.

m. After the step of digestion with uricase, neither glucose nor uric acid reduced the reagent to form color.

Fig. 1 illustrates the effect of adding increasing amounts of glucose to a constant amount of uric acid. In urine which contains a normal amount of uric acid and a 4% glucose concentration, on proper dilution, the urine aliquot would give a reading about 80% higher than could be accounted for by the amount of uric acid alone, as that aliquot would contain 4 mg of glucose.

Further study of the uricase procedure disclosed that the loss of reducing action of glucose was due to the borate buffer. When a carbonate buffer was substituted for the borate in the uricase digestion, it was found that the reducing action due to glucose was not destroyed, indicating the hydrogen ion concentration was not a factor. If borate buffer is added to mixtures of uric acid and glucose before color development, the reducing action of glucose is suppressed. This is illustrated by the data in Tables I and II which show the results obtained on the addition of borate to pure uric acid solutions as well as to urine. Loss of reducing action of glucose to alkaline copper solutions in the presence of borate was reported by Levy(2,3). Levy demonstrated that this was due to the formation of a borate glucose complex.

In conclusion, glucose in the presence of uric acid reduces arsenophosphotungstic acid to a much greater extent than it does alone. The usual conditions of uricase incubation are such that a glucose borate complex is formed with a loss of the reducing action of the glucose. Since in the method of Buchanan, Block and Christman borate is only added to the aliquot treated with uricase, but not to the one used for the total color determination, er-

TABLE I.

Effect of Glucose and Borate on the Uric Acid Determination.

(Uric acid 50 μg; glucose 4 mg; borate buffer at pH 9.2 (Clark Pg. 201) 3 ml in 50 ml of water). Read in a Coleman Junior Spectrophotometer at 660 μ.

| Contents of tube | Glucose | Uric acid | Glucose + Borate | Uric Acid + Borate | Uric Acid + Glucose | Uric Acid + Glucose + Borate |
|---|---|---|---|---|---|---|
| Total Chromogen as μg Uric Acid | 9.5 | 50. | 0. | 48.7 | 95.2 | 50. |
| After incubation of each of the above with uricase and subtraction of the uricase blank. | | | | | | |
| Residual Chromogen as μg Uric Acid | 2. | 0.5 | 2. | 0.5 | 1.5 | 1.5 |
| Apparent "True Uric Acid" | 7.5 | 49.5 | — | 48.2 | 93.7 | 48.5 |

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1. Buchanan, Block, and Christman, *J. Biol. Chem.*, 1945, v157, 181.

2. Levy and Doisy, *J. Biol. Chem.*, 1928, v77, 733.

3. Levy and Doisy, *J. Biol. Chem.*, 1929, v84, 749.

TABLE II.
Changes in Total and Residual Chromogen in Urine.
(10 ml of normal urine diluted 1:10; 4 mg glucose added, and 3 ml borate buffer in 50 ml of water).

| Contents of tube | Urine | Urine + Borate | Urine + Glucose | Urine + Glucose + Borate |
|---|---|---|---|---|
| Total Chromogen as μg Uric Acid | 53.9 | 53.6 | 97.4 | 54.4 |
| After the incubation with uricase, and borate buffer. | | | | |
| Residual Chromogen as μg Uric Acid | | | 8.6 | 8.6 |
| Apparent "True Uric Acid" | | | 88.8 | 45.8 |

roneously high values result. Addition of borate to both the aliquot treated with uricase, and to the total color determination eliminates the interference of glucose.

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## Effect of Vitamin $B_{12}$ on Experimental Hepatic Injury.* (17686)

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Observations on the effect of crude liver extract in experimental hepatic injury in rats have been previously reported(1-3). It has been shown that liver extract given by mouth is without influence on, or will even promote the development of acute diffuse hepatic necrosis. On the other hand, liver extract will not only prevent cirrhosis but has a definitely beneficial effect in the treatment of hepatic cirrhosis. Thus, the effect of liver extract may be comparable to that of choline, the latter also having a beneficial effect on cirrhosis, and no effect or a deleterious one on necrosis(1,4,5). The fact that the combination of liver extract with methionine or with protein proved to be superior in the treatment of experimental cirrhosis(3) to the effect of methionine or protein alone seemed to indicate that the beneficial effect of the liver extract might not have been due to its content of choline but perhaps to some other constituent, such as vitamin $B_{12}$. This supposition was based on the assumption that choline, methionine and protein (caesin) all act similarly as sources of "labile methyl" and it was difficult to believe that a simple increase in supply of the same factor over and above a sufficient supply could have improved the therapeutic results as distinctly as was seen on the addition of liver extract to methionine and protein.

In the course of their studies on the lipotropic effect of liver extract Hall and Drill(6) reached similar conclusions. They found liver extract a much more potent lipotropic agent than could have been expected from its content of choline alone. A more direct proof of the sparing effect of vitamin $B_{12}$ on the choline ("labile methyl") requirement of rats and chicks was first presented by Schaefer, Salmon and Strength(7). They were able to prevent "hemorrhagic kidney" in rats, and promote growth in chicks with suboptimal amounts of choline and methionine, provided $B_{12}$ was added to the diet (3 $\gamma$/100 g). This interrelationship between vitamin $B_{12}$ and methylating compounds has been confirmed on chicks by Gillis and Norris(8).

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* This represents work carried out under the auspices of the Commission on Liver Disease of the Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

1. György, P., and Goldblatt, H., *J. Exp. Med.*, 1942, v75, 355.
2. György, P., and Goldblatt, H., *J. Exp. Med.*, 1949, v90, 73.
3. György, P., *Med. Clin. N. Am.*, 1949, 1657.
4. Daft, F. S., Sebrell, W. H., and Lillie, R. D., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 1.
5. Himsworth, H. P., *The Liver and Its Diseases*, Ed. I, Oxford, England, Blackwell, Scient. Publ., 1947.
6. Hall, C. A., and Drill, V. A., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 3.

In the light of this new evidence it became desirable to study the effect of $B_{12}$ as such in various forms of liver injury, in order to be able to distinguish between the effects of choline and $B_{12}$ in liver extract.

*Massive hepatic necrosis.* Hepatic necrosis in rats fed a diet low in casein occurs but only very irregularly(1,9). A more severe, truly massive hemorrhagic necrosis of the liver can be produced in almost 100% of the experimental animals by using a diet with a proper preparation of yeast as sole source of protein(5,10-12). Yeast is free from $B_{12}$(13), whereas casein, if not especially purified, may contain traces of $B_{12}$. Thus the question arises whether a deficiency of $B_{12}$ may play a part in the development of this especially severe fulminating massive hepatic necrosis when yeast rations are used. It may even be surmised that the irregular occurrences of hepatic necrosis in rats fed casein-containing rations are related to traces of $B_{12}$ remaining in the purified casein. The often observed, slightly injurious effect of liver extract in this form of hepatic necrosis would then be due to the presence of choline.

This assumption, however, was not borne out by experiments in which the incidence of hepatic necrosis was compared in groups of rats receiving a "necrogenic" yeast ration with or without supplements of vitamin $B_{12}$. The composition of the yeast rations was as follows: A. Yeast,† 18; corn starch, 79; salt mixture (USP II), 3. B. Yeast corn starch, 59; lactose, 20; salt mixture (USP II), 3. Peanut oil, 0.5 ml and cod liver oil, 2 drops, were added for each 8 g of the dry mixture (ration A or B). All animals received daily 20 $\gamma$ "Synkayvite"‡ and supplements of 20 $\gamma$ thiamine, 25 $\gamma$ riboflavin, 20 $\gamma$ pyridoxine, and 100 $\gamma$ calcium pantothenate, dissolved together in 1 ml water. Rats were of the Sprague Dawley strain, males, weanlings, with an average weight of 50 g. Twelve rats received ration A without supplement of $B_{12}$§, 6 rats received the same ration with vitamin $B_{12}$, 1 $\gamma$ daily by mouth. Seven additional rats were fed ration B with vitamin $B_{12}$, 1 $\gamma$ daily by mouth. Thus, altogether 13 rats received a necrogenic yeast diet with supplement of vitamin $B_{12}$, and 12 rats were used as controls without $B_{12}$. All rats in both groups died from typical massive hemorrhagic necrosis, involving preferentially the left lobe or left half of the liver. There was no statistically significant difference in length of survival of the animals in the two groups; rats fed without $B_{12}$ died on the average after 34, those receiving supplement of $B_{12}$ after 36, experimental days. Thus, vitamin $B_{12}$ given by mouth was without any influence on the production of massive hepatic necrosis in rats receiving a "necrogenic" yeast diet.

*Lipotropic effect of $B_{12}$.* Experimental dietary hepatic cirrhosis is due to insufficient supply of lipotropic factors, and is accompanied, at least initially, by fat infiltration of the

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7. Schaefer, A. E., Salmon, W. D., and Strength, D. R., *Fed. Proc.*, 1949, v8, 395; PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 193, 202.

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11. György, P., *Biol. Antioxidants*, Trans. 3rd Conf., N. Y., Josiah Macy, Jr., Foundation, 1948.

12. György, P., Rose, C. S., Tomarelli, R. M., and Goldblatt, H., *J. Exp. Med.*, in press.

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† "D.C.L. Vit. $B_1$ yeast" from Distillers Co., Ltd., Glasgow C.4, Scotland.

‡ Water-soluble vitamin K preparation, Hoffmann-LaRoche, Nutley, N.J.

§ Kindly supplied by Chas. Pfizer and Co., Inc., Brooklyn, N.Y.

liver. Thus, it may be assumed that substances with lipotropic activity should have a beneficial effect on the production and perhaps even on the treatment of dietary hepatic cirrhosis. Direct evidence may only be obtained in long-term experiments in which the effect of the substance in question is studied on the prevention and treatment of hepatic cirrhosis. Such investigations on the effect of $B_{12}$ in the treatment of dietary cirrhosis of the liver are in progress.‖ The present report includes experiments designed for the study of the lipotropic activity of $B_{12}$.

The same general procedures were followed as were previously reported(14,15). Young adult female rats of the Sprague Dawley strain, weighing 145-175 g, were used. As usual, control and treated animals were run simultaneously. When all of the animals of an experiment could not be run at one time, equal numbers of the various groups were started together. Two types of rations were used: a) Low protein-high fat ($V_4$), and b) Low protein-low fat ($V_{11}$). *$V_4$ (Exp. 31):* Casein, G.B.I. Vitamin Test, 8; "Vream"¶, 40; Sucrose, 48; Salt mixture (USP II), 4. *$V_{11}$ (Exp. 33:* Casein, 8; "Vream", 5; Sucrose, 83; Salt mixture, 4. Each animal received daily 20 γ thiamine, 25 γ riboflavin, 20 γ pyridoxine, 100 γ calcium pantothenate dissolved in 1 ml of water. Three drops of percomorph oil and 3 mg of α-tocopherol were given weekly. When methionine was given, the daily dose (50 mg dl-methionine)** was dissolved in 1 ml of water and mixed with the vitamin B supplement. $B_{12}$ was administered similarly, in form of a solution contain-

TABLE I. The Lipotropic Effect of $B_{12}$. Ten rats in each experiment.

| Exp. | Diet | Treatment | Liver | | Rat weight | | Food intake, g/day |
|---|---|---|---|---|---|---|---|
| | | | Wt, g | Total fat, % | Initial, g | Change, % | |
| 31 | $V_4$ (High Fat) | Control | 7.7 ± .40 | 27.8 ± 2.6 | 156 | — 8.8 ± 3.3 | 6.1 ± .36 |
| | | $B_{12}$ | 6.7 ± .24 | 23.0 ± 2.2 | 156 | — 8.7 ± 1.2 | 5.9 ± .19 |
| | | Methionine | 6.5 ± .41 | 18.3 ± 1.4 | 152 | — 7.0 ± 1.6 | 5.5 ± .30 |
| | | Meth. + $B_{12}$ | 5.9 ± .35 | 14.7 ± 1.6 | 152 | —11.3 ± 1.8 | 5.0 ± .25 |
| 33 | $V_{11}$ (Low Fat) | Control | 9.3 ± .44 | 35.0 ± 1.7 | 160 | —12.7 ± 2.1 | 8.1 ± .36 |
| | | $B_{12}$ | 8.0 ± .56 | 20.5 ± 1.6 | 158 | — 2.6 ± 1.4 | 9.4 ± .20 |
| | | Methionine | 6.5 ± .48 | 14.6 ± 1.8 | 161 | — 8.3 ± 2.7 | 8.1 ± .43 |
| | | Meth. + $B_{12}$ | 6.6 ± .27 | 10.4 ± 1.3 | 158 | — 4.5 ± 2.9 | 8.6 ± .41 |

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‖ Investigations are also carried out at the Army Hepatic and Metabolic Center, Valley Forge General Hospital, on the metabolic and therapeutic effect of $B_{12}$ in man (normals and patients with subacute hepatitis and cirrhosis), in collaboration with Dr. V. M. Sborov and Lt. D. Sutherland.

14. György, P., Rose, C. S., and Shipley, R. A., *Arch. Biochem.*, 1947, v12, 125.

15. György, P., Rose, C. S., and Shipley, R. A., *Arch. Biochem.*, 1949, v22, 108.

¶ Vegetable shortening, obtained from Swift & Co., Chicago.

** Kindly furnished by Wyeth, Inc.

ing 0.5 γ of the crystalline compound.†† At the end of the 21 day experimental period, the animals were sacrificed and the liver analyzed for total lipide with the same technique used in the previous experiments.

The results obtained are summarized in Table I. In Exp. 31, using a low protein-high fat diet, administration of $B_{12}$ was followed by a slight statistically insignificant reduction of the relative liver fat content. When combined with methionine, $B_{12}$ had increased only slightly, without statistical significance the lipotropic effect of methionine alone. It should, however, be pointed out that the average weight of the liver, and in consequence, the absolute fat content of the liver in Exp. 31 were distinctly reduced in the groups of animals receiving supplements of $B_{12}$ (with or without methionine) to their low protein-high fat ration.

The lipotropic effect of $B_{12}$, suggested in Exp. 31, became clearly evident in Exp. 33 when the low protein-low fat diet was used. It appeared to be especially marked in the group of animals receiving $B_{12}$, without methionine, as supplement to the basal ration. The slightly increased average food intake and better average weight curve in the groups of animals receiving $B_{12}$ over those in the control groups should have, if anything, counteracted to some extent the lipotropic effect of $B_{12}$(16,17).

The lipotropic effect of a $B_{12}$ concentrate was found recently also by Drill and McCormick(18). These authors used a lipotropic diet with moderately high protein (16%) and high fat (51%) content. The $B_{12}$ concentrate was given by injection (0.2-1.0 γ, 3 times per week).

We were able to show that $B_{12}$ may exert its lipotropic effect also when given by mouth. Further, the experiments here reported indicate that a low protein-high fat diet will hinder the demonstration of lipotropic activity by $B_{12}$, whereas a low protein-low fat diet, or, as in the experiments of Drill and McCormick, a moderately high protein-high fat diet, will not interfere with the lipotropic reaction following the administration of $B_{12}$. These observations may also be related to the findings of Bosshardt, Paul and Barnes(19) on the growth-promoting effect of $B_{12}$ in mice receiving varying proportions of fat and protein in their diet. Special studies are required to answer the questions whether with increased dosage of $B_{12}$ the lipotropic effect may become evident in rats fed a low protein and high fat diet, or whether the lipotropic effect as such may be potentiated by giving higher amounts of $B_{12}$. It should be pointed out that in our experiments methionine has proved to be a more effective lipotropic agent than vitamin $B_{12}$. Experiments are contemplated to show whether additional amounts of vitamin $B_{12}$ will increase its lipotropic effect, or whether the sparing effect of vitamin $B_{12}$ could be made more evident with reduced suboptimal amounts of the lipotropic compound (choline, methionine).

*Summary*. Vit. $B_{12}$ (1 γ daily, by mouth) had no effect on the development of massive hepatic necrosis in rats fed experimental rations with yeast as sole source of protein.

Vit. $B_{12}$ (0.5 γ daily by mouth) has shown significant lipotropic activity in rats fed a low protein-low fat ration. Under the experimental conditions chosen methionine was more effective than vitamin $B_{12}$ in preventing fat deposition in the liver. A low protein-high fat diet seemed to interfere with the lipotropic effect of $B_{12}$.

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†† Kindly supplied by Merck & Co.

16. Treadwell, C. R., Tidwell, H. C., and Gart, J. H., *J. Biol. Chem.*, 1944, v156, 237.

17. Treadwell, C. R., *J. Biol. Chem.*, 1945, v160, 601.

18. Drill, V. A., and McCormick, H. M., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 388.

19. Bosshardt, D. K., Paul, W. J., and Barnes, R. H., *J. Nutrition*, in press.

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## Fradicin, an Antifungal Agent Produced by *Streptomyces fradiae*.[*] (17687)

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*Streptomyces fradiae*, the organism that produces neomycin, an antibiotic which is active upon bacteria and actinomycetes, and which has but little activity upon true fungi, also forms an antibiotic which has no activity upon bacteria and actinomycetes and is active upon fungi. This antifungal agent is a fraction of the previously noted "Factor X"(1). As much as 30 to 50% of the antimicrobial activity of the broth was found to be due to this factor. When less air was supplied during growth of *S. fradiae* in submerged culture, the concentration of this factor was increased to 66.6% in bacto-peptone broth and to 69.2 in soya peptone medium. Further studies brought out the fact that factor X is made up of two entities, one of which is an antibacterial and the other an antifungal substance. The latter has been designated as *fradicin.* It is active against a variety of fungi, including various saprophytic and pathogenic forms, such as *Penicillium notatum, Aspergillus niger, Ceratostomella ulmi, Trichophyton mentagrophytes* and *Candida albicans*, as shown in Table I. The amount of material necessary to inhibit the growth of *P. notatum* in 1 ml of nutrient agar, as determined by the agar-streak method(2) is taken as a unit. The ratio of activity against different fungi may change with the composition of the medium, possibly because of the limitations of the method. Its usual activity in culture broth is 2 to 4 times as great against *P. notatum* and *C. ulmi* as against *T. mentagrophytes* and *C. albicans*. The presence of glucose in the test medium reduces the activity of the antibiotic, either by reducing its potency or by stimulating growth of the test organisms.

TABLE I.
Antibiotic Spectrum of Fradicin.

| Organism | Units/g |
|---|---|
| *Staphylococcus aureus* | 0 |
| *Bacillus mycoides* | 0 |
| '' *subtilis* | 0 |
| *Escherichia coli* | 0 |
| *Streptomyces griseus* | 0 |
| *Trichophyton mentagrophytes* | 300000 |
| *Trichoderma* 55 | 250000 |
| *Aspergillus niger* 13 | 400000 |
| *Fusarium* sp. | 50000 |
| *Penicillium notatum* 50 | 750000 |
| *Ceratostomella ulmi* | >1000000 |
| *Candida albicans* | 250000 |

*Production and isolation of fradicin.* Media containing glucose give good production of fradicin, as shown in Table II. Addition of $CaCO_3$ is not necessary, since the medium does not become too acid even with 1% glucose. Fradicin is removed from the broth on acidification to pH 2.0-2.5 and treatment with active charcoal. Since fradicin is readily soluble in butanol at a pH range of 2.0 to 8.0, this solvent is used for further extraction. Fradicin can also be removed directly from the broth by acidification to pH 2, filtering with the aid of supercel, and eluting the supercel with sodium hydroxide solution at pH 8.0. The final isolation of fradicin from the medium and further concentration can best be carried out as follows:

Two liters of soya-peptone culture filtrate with an activity of 75 *P. notatum* units per ml is stirred for 30 minutes with one liter of *n*-butanol at pH 7.0. The alcohol phase is concentrated *in vacuo* to dryness and taken up in 95% ethanol. The alcoholic solution is concentrated to small volume and added dropwise to acetone. The solution is concentrated *in vacuo* and added dropwise to petroleum ether. The supernatant liquid phase is discarded. The oil is dissolved in *t*-butanol and lyophilized. A light tan-colored powder, hygro-

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[*] Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University,-The State University of New Jersey, Department of Microbiology.

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TABLE II.
Influence of Composition of Medium upon Formation of Fradicin.
Shake cultures, 5 days incubation.

| Medium | Dilution units per ml | | |
|---|---|---|---|
| | *P. notatum* | *C. albicans* | *C. ulmi* |
| Soya peptone | 100 | 50 | 200 |
| Nutrient broth | $<10$ | $<10$ | 50 |
| Yeast-glucose | 100 | 50 | $>200$ |
| ’’ ’’ + $CaCO_3$ | 75 | 35 | 175 |
| NZ amine | 50 | 10 | 100 |
| ’’ + 1% glucose | 100 | 65 | 200 |
| ’’ + ’’ + $CaCO_3$ | 75 | 30 | 100 |

TABLE III.
Stability of Fradicin.

| pH | Time, hr | Temp., °C | Dilution units per ml | | |
|---|---|---|---|---|---|
| | | | *P. notatum* | *C. ulmi* | *T. mentagrophytes* |
| Original potency of broth | | | 50 | 75 | 20 |
| 1.5 | 72 | 25 | 30 | 30 | 5 |
| 2.0 | 72 | 25 | 30 | 30 | 5 |
| 3.0 | 72 | 25 | 40 | 50 | 10 |
| 7.0 | 72 | 25 | 50 | 75 | 20 |
| 2.0 | 1/6 | 60 | $<5$ | $<5$ | $<5$ |
| 7.0 | 1/2 | 100 | 50 | 50 | 20 |

scopic in nature and having a potency of 312,000 *P. notatum* dilution units, is obtained. The yield is about 50 mg per liter of culture, with a recovery of 20 to 30% of the original activity.

*Solubility and stability of fradicin.* Fradicin is soluble in various alcohols (methanol, ethanol, butanols and amyl alcohols) and in chloroform. It is insoluble or very sparingly soluble in ether, carbon tetrachloride, benzene, acetone, and petroleum ether. Although only slightly soluble in water, concentrated alcoholic solutions can be dropped into a large volume of water without precipitation. Fradicin is stable at 100°C for 30 minutes in a solution of pH 7.0. At pH 2.0, 10 minutes at 60°C is sufficient to destroy the antibiotic completely. At room temperature, fradicin is stable at pH 7.0, but gradually loses its activity at acid reactions. This is brought out in Table III.

*Comparison of fradicin with other antifungal antibiotics.* Among the other antibiotics that possess strong antifungal properties, one is tempted to compare fradicin with streptothricin, on the one hand, and with actidione and antimycin A. on the other. Since streptothricin also possesses marked antibacterial properties and is so different in its solubility properties, further comparison is precluded. The solubility properties and the lack of stability of actidione above pH 7 differentiate this antibiotic from fradicin and antimycin A. Finally, the ultraviolet absorption spectrum of a sample of fradicin having a potency of 800 units per mg showed no maxima at either 227 or 330 m$\mu$ (Fig. 1); this definitely characterizes fradicin in contradistinction to antimycin A(3,4). The antimicrobial spectra of these 3 antibiotics are also markedly different.

*Summary.* An antibiotic, designated as fradicin and active only upon fungi, was isolated from the culture filtrate of the neomycin-producing *Streptomyces fradiae.* Fradicin is characterized by an antifungal spectrum, which includes saprophytes as well as plant and animal pathogens. It is stable to 100°C for 30 minutes at pH 7.0. At more acid reactions, it is gradually destroyed. It is soluble

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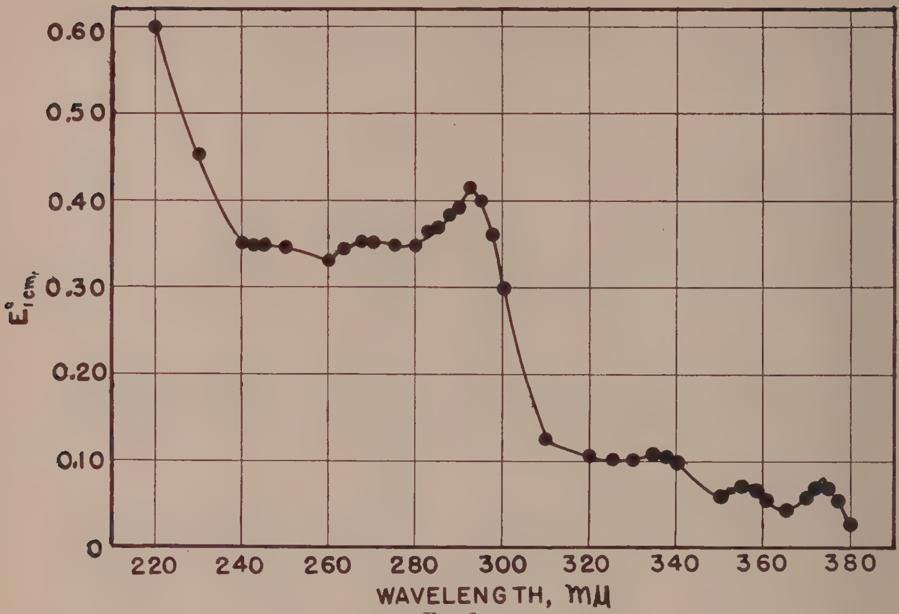

FIG. 1.
Absorption Spectrum of Fradicin.
c = 0.0174 g/l in absolute methanol.
$E^c_{1cm}$ = extinction at conc. c.

in a number of organic solvents and has a characteristic absorption spectrum which differentiates it from other antibiotics.

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## Does the Excretion of Diethylstilbestrol Glucuronide Influence the Venning Determination for Urinary Pregnanediol? (17688)

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An apparent increase in the excretion of pregnan-3($\alpha$), 20($\alpha$)-diol as a result of the prophylactic administration of diethylstilbestrol to a pregnant woman was first reported in 1946(1). This observation has been amply confirmed in this laboratory(2,3,4) and by other investigators(5), provided the pregnanediol is measured as the sodium glucuronide (NaPG) by the Venning procedure(6).

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It has also been reported, however (and confirmed in this laboratory)(4), that pregnanediol measured by colorimetric assay on acid hydrolyzed urine usually shows no increase following stilbestrol administration(5,7,8). In fact, an apparent drop has been observed in short time experiments by Marrian and Clayton upon the initiation of therapy or an increase in dosage(8). This discrepancy in results, apparent even when aliquots of the same specimens are analyzed by the two methods(4,5), introduces some question as to the validity of the interpretation of the original observation, namely, that the apparent increase in pregnanediol excretion reflects higher levels of circulating progesterone as a result of the administered diethylstilbestrol.

It is now known that recrystallized NaPG prepared from human urine by the Venning method may contain the sodium glucuronides of substances other than pregnanediol. This statement is based upon the identification of three compounds, pregnane-3(α), 17, 20-triol(9), pregnane-3(α), 17-diol-20-one(10), and pregnane-3(α)ol-20-one(11) as glucuronides in "purified" NaPG complex from human urines. The latter is present in considerable amounts in pregnancy urine(11) and has been shown to be an excretory product of progesterone(12). No evidence has been presented that diethylstilbestrol glucuronide is recovered by the Venning procedure but the possibility has naturally arisen(5,8) since, from the urines of both rabbits(13) and women(14,15) to whom stilbestrol has been administered, diethylstilbestrol glucuronide has been obtained in quantities amounting to 6 to 35% of the stilbestrol given. If the glucuronide of diethylstilbestrol were recovered by the Venning procedure, therefore, its presence in the NaPG crystals might account for the apparent effect of stilbestrol administration upon pregnanediol excretion. In this communication evidence is presented which eliminates this possibility. Some other explanation must be sought, therefore, for the discrepancy in results for "pregnanediol" excretion, and investigations along this line are in progress(16).

*Estrogenic activity of NaPG from the urine of pregnant women taking diethylstilbestrol by mouth.* Four samples of crystalline NaPG recovered by the Venning procedure from the urines of 4 women, normally pregnant at weeks 34 to 38, were tested for estrogenic activity. All of the women had been on oral therapy with gradually increasing dosages of stilbestrol from early in pregnancy. At the time when these samples of NaPG were recovered they were taking 125 to 150 mg of the drug daily.

The first sample (m. p. 282°C) was dissolved in isotonic buffer solution and tested directly, without any hydrolysis or extraction. In 10 spayed female rats, standardized for their responsiveness to crystalline estrogens, the vaginal smears were completely negative when testing for 10 rat units in 15 mg. By our assay 2.5 µg of diethylstilbestrol glucuronide is equivalent to one rat unit. It may be assumed, therefore, that this sample contained less than 0.08 mg of diethylstilbestrol glucuronide in the 45.8 mg of NaPG recovered, an amount of estrogenic impurity well below the degree of accuracy of the Venning method.

The other 3 samples were dissolved either in concentrated phosphate buffer solution or postmenopausal urine, subjected to acid hydrolysis and the phenolic fractions recovered by ether extraction and partition out of toluene with normal sodium hydroxide. The method of extraction used has been shown to

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give a quantitative recovery of crystalline diethylstilbestrol, but the method of hydrolysis (10 minute boiling with 15 volumes percent of concentrated hydrochloric acid) accomplishes only 50% hydrolysis of diethylstilbestrol glucuronide. In calculating from the estrogenic activity recovered the possible contamination of the NaPG with stilbestrol glucuronide, therefore, a 50% loss was assumed.

An 80 mg sample (m. p. 268 to 275°C) was found to contain 50 to 67 r.u. of estrogenic activity after hydrolysis and extraction. By our assay, 0.15 $\mu$g of diethylstilbestrol is one rat unit. This sample, therefore, could have contained no more than 0.038 mg of sodium diethylstilbestrol glucuronide, too little to influence the gravimetric analysis.

A 55 mg sample (m. p. 275 to 276°C) treated in the same manner yielded 22 to 30 r.u. of estrogen. It could have contained no more than 0.017 mg of sodium diethylstilbestrol glucuronide, too little to be detected gravimetrically.

A 60 mg sample (m. p. 282°C) was subjected to our modification of the Sommerville, Gough and Marrian method for the hydrolysis and extraction of urinary pregnanediol(16). According to the estrogenic activity recovered from the phenolic fraction, the highest possible concentration of sodium diethylstilbestrol glucuronide in this NaPG was 0.022 mg, again too little to measure with gravimetric accuracy.

*Failure to recover NaPG from the urine during the administration of diethylstilbestrol.* Results have been previously reported(2) showing that the oral administration of 75 to 125 mg of stilbestrol daily to patients already showing the clinical signs of late pregnancy toxemia causes no increase in pregnanediol excretion (as measured by the Venning procedure). This observation has been repeatedly confirmed in this laboratory. A recent case specifically illustrates the point. The patient was 26 weeks pregnant with fulminating severe pre-eclampsia. Three specimens of urine collected on 3 consecutive days before stilbestrol was started were found to contain 8.2 to 11.0 mg of pregnanediol as NaPG per 24 hour volume. During the following 10 days she was on 100 mg of diethylstilbestrol daily by mouth. Four urines were analyzed and found to contain 9.3 to 11.7 mg of pregnanediol as NaPG per 24 hour volume, no increase over the pretreatment level. In 3 unpublished experiments it was also found that 125 to 200 mg of stilbestrol daily would not prevent the drop in NaPG that precedes normal labor at term.

An obese diabetic woman thought to be pregnant by her obstetrician was put on 35 mg of diethylstilbestrol daily at the seventeenth week from her last menstrual period. Her urines were analyzed for pregnanediol by the Venning procedure at weekly intervals. No glucuronide was recovered in any of 7 specimens, the last one having been collected at 25 weeks when 75 mg of stilbestrol were being ingested daily. At this time the obstetrician confirmed our opinion that the patient was not pregnant.

To another non-pregnant woman 100 mg of diethylstilbestrol was administered experimentally. No NaPG was recovered by the Venning procedure from the butyl alcohol extract of all of the urine collected during the following 72 hours. A mature female rabbit was injected with 200 mg of crystalline diethylstilbestrol and the urine for the next 72 hours extracted by the Venning procedure. No NaPG was recovered.

*Recovery experiment with diethylstilbestrol glucuronide added to urine.* A sample of diethylstilbestrol glucuronide was prepared by one of us (S. S.) from the urine of a rabbit that had received 1.5 g of crystalline diethylstilbestrol by injection over a 7 day period. The procedure described by Mazur and Shorr (13) was followed. Repeated recrystallization was resulting in so much loss of material that complete purification was not attempted. The sample used crystallized in white needles, softened at 91 to 96°C and melted with decomposition at 132 to 170°C. Tests for estrogenic activity before and after hydrolysis indicated that this was practically pure diethylstilbestrol glucuronide. Prior to hydrolysis it assayed at 1 rat unit to 2.5 $\mu$g. An aliquot was hydrolyzed according to the method of Teague(15), after which the activity was increased to 1 rat unit to 0.45 mg, *i.e.,* 1 rat unit to 0.25 μg of free diethylstilbestrol, the same assay value acquired from a sample of crystalline diethylstilbestrol which was simultaneously submitted to the same hydrolysis procedure.

Forty mg of this sample were dissolved in 200 cc of postmenopausal urine and extracted with butyl alcohol for the recovery of NaPG by the Venning method. No precipitate whatsoever settled out of the aqueous acetone solution. Estrogenic assay of the various fractions revealed that half of the stilbestrol glucuronide had been left in the tenth normal NaOH solution after being re-extracted with butyl alcohol. The other half was present in the residue of the final butyl alcohol extract but, unlike pregnanediol glucuronide, was not precipitated out of 5 cc of water by the addition of 95 cc of acetone. It is quite possible that when considerable amounts of NaPG are present, traces of stilbestrol glucuronide may be carried down by adsorption and even persist through the second precipitation out of water with acetone. That no more than traces are present, however, is apparent from the very slight estrogenic potency of NaPG recovered from pregnant women taking large doses of stilbestrol (v.s). In this connection it may be mentioned that estrogenic activity has also been found by us in hydrolyzed NaPG recovered from the urine of pregnant women who were not taking diethylstilbestrol. The probable explanation is that traces of estriol glucuronide, large amounts of which are present in late pregnancy urine, may also be carried down by adsorption upon the precipitated NaPG. Here again the amount present, although readily demonstrable by estrogenic assay, is too little to influence the figures for pregnanediol recovered as NaPG.

*Conclusion.* Diethylstilbestrol glucuronide is recovered in no more than trace amounts from human urine by the gravimetric Venning procedure for the determination of sodium pregnanediol glucuronide. In pregnant patients taking large amounts of stilbestrol the quantitative values for urinary pregnanediol were not influenced by this possible source of error. Some other explanation must be found for the fact that values acquired by the Venning method on the urines of such patients do not agree with colorimetric assays for "freed" pregnanediol upon hydrolyzed specimens.

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## Activity of Trypsin in Solution Through Membranes.* (17689)

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The concept of specific long range forces of certain biologically active materials has been promulgated by Rothen(1-7). His experiments employing films of antigen and antibody, or films of enzyme and subtrate, separated by inert film barriers indicate the possible existence of such forces. The evidence thus far presented is the increase in thickness of the layered films when homologous antigen-antibody systems are allowed to interact through the barriers. Similarly, trypsin acting through a barrier on bovine albumin prevents an increase in thickness when an antiserum to bovine albumin is subsequently added. Rothen also finds that increasing the number of layers of barrier be-

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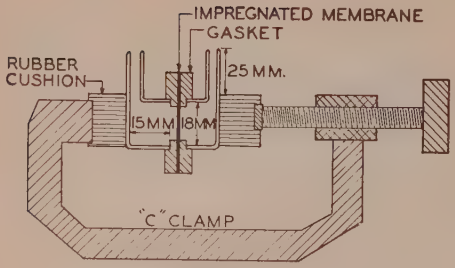

Fig. 1.
Membrane and cell assembly.

yond certain thicknesses obliterates the interacting effect. Increasing the number of monolayers below the barrier reinforces the interaction. A number of systems have all been studied by the same technique, the measurement of increment in film thickness by means of the ellipsometer(2). The findings, however, have met with considerable reservation, principally on the assumption that interaction may take place through minute holes in the film barriers(8,9). Although many substances are known to form films without holes(10) possible imperfections cannot be detected by the ellipsometer. A method for testing interaction, therefore, which might also detect leaks in the barrier could help to determine if the effect is caused by simple diffusion. Such a method, involving a trypsin solution separated by an impregnated paper membrane from a hemoglobin substrate solution, has been devised.

*Experimental. Materials and Methods:* A small 2-compartment cell was constructed with a membrane separating the compartments (Fig. 1). Each cell half was made from an 18 mm I. D. pyrex test tube. The round bottom of the tube was flattened, and a 25 mm side arm of 4 mm I. D. pyrex tubing was sealed in at a right angle to the test tube and flush with its flattened bottom. The body of the tube was cut off, and the cut edge of the cell half was ground down with carborundum so that the distance from the flat bottom to the ground edge was 15 mm. The capacity was about 3.5 ml. The second cell half was similarly constructed. Two rubber gaskets were used to hold the membrane between the cell halves. They were each constructed of 2 sheets of U. S. Rubber Company Rainbow rubber 1.5 x 35 x 35 mm. Centered circular holes were cut in each of 2 of the pieces of rubber so that the hole in one piece was of the inside diameter of a cell half, while that in the second piece was of the outside diameter. The latter fit the cell half tightly when placed over it. The 2 rubber pieces were washed with benzene and cemented together with rubber cement, the holes placed concentrically. This provided a gasket which fit over the smooth, outside edge, and flush against the ground edge, of the cell half. Another identical gasket was made for the second cell half. When the gaskets were fitted to the cell halves a membrane could be placed between them. The gaskets were conveniently held together with two 1¼-inch screw clamps over their extended edges. The screw clamps thus facilitated placing the assembled cell horizontally in a 2-inch "C" clamp. Two cushions made from rubber stoppers were cemented to the "C" clamp so that the cell could be tightened in the clamp without breaking, the flat ground edges of the cell halves pressing the membrane between the gaskets.

Membranes capable of withstanding even the small hydrostatic pressure of about 2 cm $H_2O$ when only one section of the cell was filled proved difficult to make. Membranes of formvar sufficiently thin to give interference rings (0.1-0.5% in ethylene dichloride) were cast on water and floated onto a supporting screen of 200 mesh stainless steel. However, these were found incapable of supporting the necessary pressure. Similarly collodion and parlodion also failed. It became apparent that a finer mesh for the supporting screen was required, and various types of paper were tried. Several dozen filter paper types had insufficient wet strength or were too thick. It was finally found that Esleeck onion skin typewriter paper had the necessary properties. The paper was washed overnight in distilled water, allowed to dry and cut into 2.5 cm squares.

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TABLE I.
Activity Through Membranes of Solutions of 0.1% Trypsin on 1.75% Hemoglobin.

| | | | | Mg tyrosine released | | |
|---|---|---|---|---|---|---|
| | | | | Controls | | |
| Membrane | Solvent | % (wt) | T°C | Before | After | Experimental |
| Intact: | | | | | | |
| Formvar | $C_2H_4Cl_2$ | .24 | 25 | 0 | .002 | .160 |
| Cellophane | — | .02 (mm) | 25 | .002 | 0 | 0 |
| Parlodion | $C_3H_6O$ | 2.5 | 25 | 0 | 0 | .056 |
| Collodion | ,, | .19 | 23 | 0 | .002 | .102 |
| ,, | ,, | .38 | 23 | 0 | 0 | .080 |
| ,, | ,, | 1.27 | 23 | 0 | 0 | .070 |
| ,, | ,, | 3.40 | 23 | 0 | 0 | .032 |
| ,, | ,, | 6.90 | 23 | 0 | 0 | .023 |
| ,, | ,, | 10.4 | 23 | 0 | 0 | .020 |
| ,, | ,, | 10.8 | 23 | 0 | .001 | .021 |
| ,, | ,, | 11.2 | 23 | 0 | 0 | 0 |
| ,, | ,, | 12.6 | 23 | 0 | 0 | 0 |
| — | — | — | 23 | — | — | 2.210 |
| Defective: | | | | | | |
| Cellophane | — | .02 (mm) | 22 | .008 | .006 | .020 |
| Collodion | $C_3H_6O$ | .19 | 23 | .007 | 0 | .111 |
| ,, | ,, | 10.4 | 23 | .008 | .014 | .035 |
| ,, | ,, | 11.2 | 23 | .008 | .001 | .018 |

Collodion was used predominantly in the experiments. About 100 ml Mallinckrodt U.S.P.XIII collodion was poured slowly into 2 l distilled $H_2O$ with vigorous stirring. The precipitate was washed with several changes of distilled $H_2O$ to remove most of the alcohol and ether. After drying in air overnight, it was cut in small pieces and dried to constant weight in a vacuum desiccator over $CaCl_2$ at room temperature. A series of approximately 10 ml solutions of collodion in acetone varying from 0.19-12.6% by weight was prepared. The solvents for Mallinckrodt parlodion and Shawinigan formvar were acetone and ethylene dichloride respectively. The membranes were made by immersing the paper squares in the solutions overnight. The impregnated paper was removed from the solution, the excess solution was quickly pressed out between two microscope slides, and the membrane was allowed to dry at least 30 minutes before being assembled in the cell. A new membrane was used for each test.

The trypsin used was a dried, commercial preparation. Since the enzyme best maintains its activity in the dry state, it was made into solutions of 1 mg/ml in the same vehicle as the substrate just prior to use. The vehicle consisted of 8.0 ml 1N NaOH, 40 g urea, 82 ml $H_2O$ and 10.0 ml M $KH_2PO_4$, pH 7.5(11). The activity was such that color was liberated equivalent to 2.210 mg tyrosine/ml in the presence of "phenol" reagent after digestion for 30 minutes at 23°C with the hemoglobin substrate (Table I). The enzyme activity was measured with a Klett-Summerson colorimeter at 600 m$\mu$ employing the "phenol" reagent of Folin and Ciocalteau(13) and was read from a tyrosine standard curve. Hemoglobin substrates of a final concentration of 1.75% in the same vehicle as that used for the trypsin, were prepared by urea denaturation according to Anson(11,12).

Controls for determining whether or not the membranes were intact were run on each membrane just before and after every determination. These are described in the following section. However, since only 0.5 ml of the control solutions (*vs.* 2.5 ml hemoglobin solution) could be tested, a further test for the detection of membrane leaks was made. This consisted in placing enzyme and sub-

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strate on opposite sides of a 1.27% collodion membrane in the assembled cell. After 30 minutes, 2.5 ml of substrate were removed via syringe and mixed with 8.5 ml of fresh substrate. Had enzyme leaked and been present in the substrate, it would have been expected to continue to liberate color producing substances so that the tyrosine value would increase with time. Periodically 2.5 ml of substrate were removed and tested with phenol reagent. However, in 2 experiments with different membranes there was no increase in the tyrosine value up to 180 and 250 minutes.

*Procedure.* After assembling the cell with the desired membrane, it was tightened in the "C" clamp which was supported horizontally on a ring stand. One cell half was filled with about 3.0 ml control buffer solution so that it reached a few millimeters into the side arm. The solution was introduced by means of a hypodermic syringe with stainless steel cannula. The other cell half was similarly filled with enzyme solution to the same level. The cell was allowed to stand 30 minutes when the control solution was removed by syringe to be later tested for possible enzyme which might have leaked through the membrane. The now empty half cell was filled with the hemoglobin substrate to the same level as the enzyme solution with a clean syringe and cannula. After 30 minutes during which interaction could take place, it was removed to be tested for color producing compounds. The residual hemoglobin in the cell half was gently rinsed out and discarded, and the cell half was again filled with buffer. This was allowed to stand for 30 minutes and was then removed via syringe to be tested with the first control for enzyme activity. The whole procedure was carried out at room temperature, which was maintained at 23°±0.5°C. To 0.5 ml of each of the control samples in a 20 x 150 mm test tube, 2.5 ml of fresh hemoglobin substrate were added. The two controls were allowed to incubate for 30 minutes at room temperature to test for enzyme which might have leaked through the membrane. At this time to 2.5 ml of the hemoglobin which had been in contact with the membrane in the same size tube, 0.5 ml of buffer was added. This brought all three solutions to the same concentrations. A reagent blank consisting of 2.5 ml of hemoglobin substrate and 0.5 ml of buffer had also been prepared at the time of introduction of hemoglobin substrate into the cell. At the conclusion of the 30 minute digestion period, 5.0 ml 0.300 N trichloracetic acid were quickly added to each of the 4 samples, and they were filtered to remove the precipitated hemoglobin. To 5.0 ml of each filtrate were added 10.0 ml 0.5 N NaOH and 3.0 ml of "phenol" reagent. The samples were read in 2-15 minutes in the colorimeter with the instrument set at 0 with the reagent blank. The standard deviation, $\sigma$, for the tyrosine standard curve was 0.0050 mg tyrosine per ml.

*Results.* Table I illustrates the results obtained with several different membrane materials made at various concentrations. In the experiments employing 0.24% formvar, 2.5% parlodion and 0.19-10.8% collodion membranes, the trypsin acted on the hemoglobin substrate to liberate color producing substances. There was no indication that the membranes allowed trypsin to leak into the control solutions during the 30 minute control periods before and after enzyme and substrate had been in contact with the membranes. These membranes are labeled "intact." The values for several "defective" membranes are included in Table I. Since the standard deviation for the tyrosine standard curve was 0.0050, experiments in which the control values were greater than this were considered to have defective membranes. Fig. 2 illustrates the effect of increasing the collodion concentrations in a series of membranes from 0.19-12.6%. It was found that the tryptic action on the hemoglobin follows a decreasing, non-logarithmic, curvilinear pattern up to 11.0% collodion. At this point the effect abruptly drops from 0.020 mg tyrosine per ml to zero.

*Discussion.* Interpretation of the results reported here is necessarily centered about the controls since evidence for interaction across an inert membrane must preclude the possibility of enzyme and substrate diffusing into one another through imperfections in the membrane. That they do not diffuse through

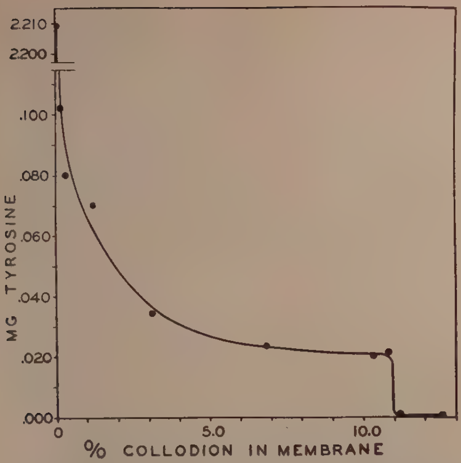

FIG. 2.

Decrease in trypsin activity as the concentration of collodion in the membranes is increased. Trypsin activity is expressed as mg of tyrosine.

the membranes is shown by the controls.

The high degree of sensitivity of the controls is illustrated in Table I from the differences between the same kind of intact and defective membranes. Thus, in the case of the cellophane membranes, the intact membrane transferred no tryptic activity, nor did the controls indicate leaking. However, the defective membrane showed a tryptic activity value of .020, while its controls jumped to .008 and .006 indicating leakage. Similar results were obtained for intact and defective 0.19, 10.4 and 11.2% collodion membranes. Therefore, experiments yielding no leak values but yielding transfer values from .020 all the way to .160 must require the interpretation that tryptic activity had been transferred across the membranes in some manner. In Rothen's experiments the thickness of the films cast on smooth surfaces may be measured with considerable accuracy by the ellipsometer. The thickness of the films in the interstices of the impregnated membranes is purely a matter of conjecture. However, both methods indicate transference of tryptic activity across thin membranes. Rothen's findings indicate that interaction takes place in systems wherein enzyme, substrate and barrier are immobilized as solid films. The experiments here described indicate that the effect also takes place when enzyme and substrate are in solution and are separated by a solid membrane. Whatever the mechanism for the transfer of the activity may be, the latter experiments show that this effect would have to be ascribed to some phenomenon other than simple diffusion through the membranes.

*Summary and Conclusions.* Formvar, parlodion and collodion membranes have been prepared by impregnating paper with solutions of these substances. A suitable cell of two compartments was constructed in which solutions of trypsin and hemoglobin could be separated by the membranes.

Interaction between solutions of trypsin and hemoglobin separated by formvar, parlodion or collodion membranes has been demonstrated.

Examination of a series of membranes impregnated with collodion of different concentrations showed that interaction decreased in a curvilinear fashion as the collodion concentration in the membranes was increased.

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## Antigenic Property of Renal Cortex.* (17690)

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The experimental production of chronic renal disease in rats by means of heteronephrotoxins obtained from rabbits has been studied by Smadel, Swift and Farr(1-3), and later

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* Aided by a grant from the Life Insurance Medical Research Fund.

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TABLE I.
Nephrotoxicity of Sera Derived from Rabbits Treated with Extracts from Kidneys, Renal Cortex, and Renal Medulla Obtained from Rats.

| Whole kidneys | | | Renal Cortex | | | Renal Medulla | | |
|---|---|---|---|---|---|---|---|---|
| No. of rats used | Amt of serum producing severe disease, cc | Nephrotoxicity of serum | No. of rats used | Amt of serum producing severe disease, cc | Nephrotoxicity of serum | No. of rats used | Amt of serum producing severe disease, cc | Nephrotoxicity of serum |
| 7 | ? | — | 8 | ? | — | 7 | ? | — |
| 7 | ? | — | 4 | .9 -1.15 | + | 7 | ? | — |
| 9 | .85-1.2 | + | 12 | .77-1.0 | ++ | 7 | ? | — |
| 14 | .77-1.0 | ++ | 3 | .5 - .7 | +++ | 7 | ? | — |
| 10 | .77-1.0 | ++ | 7 | .51- .7 | +++ | 6 | ? | — |
| 15 | .51- .7 | +++ | 6 | .38- .5 | +++ | 15 | ? | — |
| 5 | .38- .5 | +++ | 3 | .38- .5 | +++ | 5 | 1.5-2.1 | (+) |
| 7 | .38- .5 | +++ | 18 | .3 - .4 | +++ | | | |
| 12 | .38- .5 | +++ | 4 | .2 - .3 | ++++ | | | |
| 19 | .19- .26 | ++++ | | | | | | |

on by Heymann and Lund(4-5). The former investigators described this disease as a diffuse chronic and progressive glomerulonephritis while Heymann and Lund believe that it simulates lipemic nephrosis, with and without glomerular lesions(6) as observed in infants and children. Regardless of these differences in the concept of nomenclature and classification of this disorder, studies were undertaken to determine whether renal cortex and renal medulla differ in their antigenic properties.

*Methods.* Renal extracts were prepared from perfused rat kidneys according to the technic previously described(4,5). The separation of cortex and medulla was approximate and was done by sharp dissection. Rabbits were injected with 10 cc of these extracts intraperitoneally every other day. 10%, 15% and 20% extracts were used for the first 3 treatments, and 20% suspensions were used for 2 more weeks. The animals were bled 6 - 7 days after the last extract injection. Rats of the Long-Evans and Whelan strains were used. They were kept on Friskies. They were injected intravenously, when 4 - 5 weeks old and weighing 50 - 70 g, with 1/3rd of the dose on 3 consecutive days. The serum had been inactivated for ½ an hour at 56°C. The amount of serum was gauged according to the estimated kidney weight as previously described(5). The dose was then varied until the amount had been established that produced a "severe" nephrotic disease. A severe renal disease, the term used in Table I, was assumed when the majority of the injected rats showed ascites and edema at onset and massive 4+ albuminuria which did not subside for 8 weeks.

*Results.* From 26 rabbits used, 10 were treated with extracts prepared from whole kidneys, 9 with extracts from renal cortex and 7 were injected with extracts prepared

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2. Smadel, J. E., *J. Exp. Med.*, 1937, v65, 541.

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4. Heymann, W., and Lund, H., *Science*, 1948, v108, 448.

5. Heymann, W., Lund, H. F., Gilkey, C., and Salehar, M., Lipemic Nephrosis in Rats.

6. Heymann, W., and Alperin, L. J., *Am. Practitioner*, 1949, v3, 650.

from renal medulla obtained from rats. The sera of these rabbits were injected intravenously in 224 rats. It can be seen (Table I) that 8 of 10 sera obtained from rabbits treated with kidney extracts were nephrotoxic. The amount of serum needed to induce severe renal disease was from 0.19 to 1.2 cc. 8 of the 9 sera obtained from rabbits treated with cortex extracts were nephrotoxic. The dose needed to induce severe renal disease was from 0.2 to 1.15 cc. From 7 sera obtained from rabbits treated with medulla extracts only one was slightly nephrotoxic. The amount of serum needed to induce severe renal disease with that serum was greater than any of the least nephrotoxic sera of the kidney or cortex group. The remaining 6 medulla sera were tested in doses of 2 to 3 cc and no proteinuria was noted in any of the rats. The clinical course was carefully studied in all animals. The systolic blood pressure was taken every 2-3 weeks. B.U.N. determinations were carried out repeatedly intra vitam, and histological examinations of the kidneys and blood chemical studies were obtained at time of death, which included B.U.N., creatinine, serum proteins, cholesterol and total lipids. No differences were noted whether renal disease had been induced by using sera obtained from rabbits treated with whole kidney, cortex or medulla extracts. Two additional rabbits were treated with medulla extracts that were diluted in a ratio of 2 to one with 20% extracts of perfused rat spleens. The sera of these two animals did not produce proteinuria in 8 rats.

*Discussion.* The results indicate that the antigen of renal tissue is localized in the renal cortex. The nephrotoxicity of serum obtained from one of seven rabbits treated with medulla extracts may well be due to the fact that the separation of cortex and medulla was only approximate. Medulla extracts might at times have contained some cortex tissue. These results are in accordance with those obtained by Pressman, Hill and Foote(7), who studied radio autographs of kidneys after the injection of radio-iodinated globulin of anti-mouse-kidney sera. They showed localization of $I^{131}$ in the glomeruli. The observations also are consistent with results reported lately by Solomon, Gardella, Panger, Dethier and Ferrehee(8) that nephrotoxins against rat kidneys can be removed by adsorption with suspensions of rat glomeruli, but not by adsorption with other portions of rat kidney. The specificity of this phenomenon remains, however, questionable as the adsorption of other than renal antibodies was not included in that study.

The histology of glomerular tissue shows an intimate mixture of tufts of capillary loops with epithelial elements probably deriving from structures of the proximal convoluted tubules. As the sera of rabbits treated with a mixture of medulla and spleen extracts were not nephrotoxic, and considering the highly differentiated functional state of the glomerular capsule and the proximal convoluted tubules, the latter structures might be more likely to harbor the antigen than the tufts of capillary loops.

*Summary.* 1. Ten rabbits were treated with extracts of bloodfree rat kidneys. Eight of the sera obtained from these rabbits induced severe renal disease in rats, weighing 50-70 g, when injected in a dose of 0.19 to 1.2 cc.

2. Nine rabbits were treated with extracts of renal cortex obtained from rats. Eight of the sera obtained from these rabbits induced severe renal disease in rats, weighing 50-70 g, when injected in a dose of 0.2-1.15 cc.

3. Seven rabbits were treated with extracts of renal medulla obtained from rats. Only one of the sera obtained from these rabbits induced severe renal disease in rats weighing from 50-70 g when injected in a dose of 1.5-2.1 cc.

4. It is concluded that the antigen of renal tissue is localised in the renal cortex.

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7. Pressman, D., Hill, R. F., and Foote, F. W., *Science,* 1949, v109, 65.

8. Solomon, D. H., Gardella, J. W., Paugh, H., Dethier, F. M., and Ferrehee, J. W., *J. Exp. Med.,* 1949, v90, 267.

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## Effect of Growth Hormone on Temporal Muscle of the Guinea Pig.* (17691)

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The ability of testosterone propionate to stimulate growth of the temporal muscle of the castrated male guinea pig(1) has been confirmed and extended to other steroids(2). Thus another site of protein synthesis under androgen stimulation has been established. Since the growth hormone of the anterior pituitary also is a powerful stimulator of protein anabolism(3,4), it seemed worthwhile to determine whether this hormone could also stimulate growth in the temporal muscle of the guinea pig. Certain definite differences in the protein anabolic actions of androgens and growth hormone have already been demonstrated(4-6).

*Procedure*. The guinea pigs were of the Hartley strain and were obtained at 325-375 g body weight from the Tumblebrook Farms. They were placed in individual cages and fed Rockland guinea pig diet containing vitamin C *ad libitum*. Once per week each animal was given 12.5 mg of vitamin C by mouth. When the guinea pigs reached 395-405 g body weight they were castrated. Thirty to 35 days later the growth hormone was injected subcutaneously at approximately 5 p.m. of each day for 14 days. On the morning of the 15th day the animals were killed and the seminal vesicles and prostates and the temporal muscles were removed and weighed on a Roller-Smith torsion balance. The guinea pigs were weighed 3 times per week beginning on arrival and also at time of autopsy. Any animal that did not show a good growth response was discarded. The growth hormone was provided as a powder and had been prepared by the method of Wilhelmi, *et al.*(7). Assay in the 6-month-old "plateaued" female rat† gave 1288 units per gram and in the hypophysectomized immature female rat,‡ 0.1 mg/day for 10 days produced an average increase in body weight of 16.6 g. It showed no corticotrophic or gonadotrophic activities but did repair the thyroid of the hypophysectomized rat at a total dose of 2.5 mg divided in 4 daily injections. The powder was prepared for injection as previously described except that at the 2 mg and 4 mg/day dose level the concentration of the hormone was increased from 10 mg to 20 mg/ml. The lowest dose of the growth hormone preparation produces a maximal protein anabolic effect in castrated and hypophysectomized rats(4).

*Results*. The experiments were run in 2 groups (Table I). The 1 mg/day dose was run simultaneously with control-1 and the other two dose levels with control-2. The values of the 2 control groups are presented separately with ranges of the respective values.

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* This investigation was supported by a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council.

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6. Kochakian, C. D., and Stettner, C. E., *Am. J. Physiol.*, 1948, v155, 262.

7. Wilhelmi, A. E., Fishman, J. B., and Russel, J. A., *J. Biol. Chem.*, 1948, v176, 735.

† The growth hormone powder was supplied and assayed by Parke, Davis and Co. through the courtesy of Drs. D. A. McGinty and L. A. Donaldson.

‡ These assays were performed by the Institute of Experimental Biology through the courtesy of Dr. C. H. Li.

TABLE I.

Effect of Anterior Pituitary Growth Hormone on Castrated Male Guinea Pig.

| | Guinea pigs No. | Dose, mg/day | Body wt Initial, g | Body wt Change, g | Temporal muscles, mg | Sem. ves. + pros., mg | M/A,* mg/mg | Adrenals, mg | Thyroid, mg |
|---|---|---|---|---|---|---|---|---|---|
| Control 1 | 7 | | 508 | +62<br>(35-101) | 693<br>(566-729) | 552<br>(395-644) | | | |
| ,, 2 | 7 | | 570 | +45<br>(15-88) | 686<br>(603-712) | 547<br>(499-697) | | 264 | 121 |
| Growth Hormone | 7 | 1 | 543 | +30 | 730 | 606 | .71 | 241 | 118 |
| ,, | 4 | 4 | 586 | +39 | 817 | 743 | .66 | 313 | 131 |
| ,, | 4 | 4 | 586 | +39 | 818 | 684 | .95 | 288 | 141 |

* M/A (myotrophic-androgenic ratio) is the growth of the temporal muscles divided by the growth of the seminal vesicles and prostates.

*Body weight.* The growth hormone at the various dose levels did not significantly increase the rate of growth over that already present.

*Myotrophic Property.* A small increase in muscle size was produced by the growth hormone at 1 mg/day. The increase was significantly enhanced by doubling the dose but no further increase was obtained on a four-fold increase of the dosage.

*Androgenic Property.* The seminal vesicles and prostates increased in parallel with the temporal muscles.

*Adrenals and Thyroid.* The changes in these organs were equivocal.

*Discussion.* The failure to note a change in the body weight of the guinea pigs injected with growth hormone is not too unexpected. The effect of the growth hormone on body weight also is not readily apparent in growing rats especially males(8). The effect of the androgens, too, is apparent in the excised muscles before it is on the total body (2, also unpublished). The small effect on the prostate and seminal vesicles of the growth hormone preparation probably is not a true androgenic response but part of the general effect of growth hormone on all tissues. On the other hand, it is possible that trace impurities in the growth hormone preparation stimulated the production of "Albright's N-Hormone"(9) from the adrenal cortex to bring about the parallel effect on the muscle and the accessory sex organs.

The effect produced by the growth hormone at 2 and 4 mg/day is comparable to those produced by testosterone in oil solution at 0.5 mg/day but less than at 0.1 mg/day in crystalline suspension (unpublished, *cf* 2).

*Summary.* An anterior pituitary growth hormone preparation containing some thyrotrophic hormone produced a small increase in size of the temporal muscle of the castrated male guinea pig accompanied by a parallel increase in the accessory sex organs.

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# Mineralization of the Growing Tooth as Shown by Radio-Phosphorus Autographs.* (17692)

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The deposition of radio-phosphorus in growing molar teeth was examined in young hamsters with the help of the autographic technic. An injection of carrier-free radio-phosphorus ($P^{32}$) was given at the age of 4 days, that is, soon after the appearance of dentin and enamel in the germs of the first and second molars. The animals were sacrificed 1 hour, 6 hours, 1 day, 2 days or 4 days later. The jaws were fixed in a neutral formalin-alcohol mixture, embedded in celloidin without decalcification, sectioned as usual, autographed by coating the sections with photographic emulsion according to the "inverted method"(1), and stained with basic fuchsin. Dentin and enamel, but not soft tissues, showed autographic reactions. These were attributed to the presence of radio-phosphorus in a form sufficiently insoluble to be retained in the sections during the histological procedure. Presumably, the radio-phosphorus had become part of the complex phosphate salt making up the crystalline structures of the tooth (apatite?).

At 1 and 6 hours after injection, the autographic reactions were of 2 kinds: *diffuse* reactions extending throughout dentin and enamel, and *localized* reactions restricted to definite parts of these structures. The presence of diffuse reactions was in itself proof that the circulating phosphate must have been able to enter dentin and enamel. Immature enamel is known to be permeable to dyes and other solutions(2), and dentin to radio-sodium(3) and radio-iodine(4). These findings indicate that the "extra-cellular" spaces extend throughout these structures and provide the pathway for the diffusion of the circulating phosphate. Presumably, the radio-phosphorus responsible for the diffuse reactions was deposited by a process of "exchange" between the labeled phosphate circulating in the extracellular spaces and the surface phosphate groups of the crystals making up dentin and enamel(5). The gradual decrease and disappearance of the diffuse reactions, which was observed at 1, 2 and 4 days after injection, would then be due to an exchange in the reverse direction, as the labeled phosphate of the crystal surfaces was replaced by the circulating phosphate which, by that time, no longer contained significant amounts of radio-phosphorus.

At one day after injection, the decrease in the diffuse reactions caused the localized reactions to appear more prominent. In the cross-sections of dentin, the reactions were in the form of a dark line, limited to the inner dentinal region, close to the odontoblasts. At 2 days, the reaction line was in the substance of the dentin at some distance from the odontoblasts; at 4 days, this distance was approximately doubled (Fig. 1). Obviously, the reaction line in Fig. 1 was due to the radio-phosphorus incorporated in the dentin formed during the first 24 hours after injection, while the light area separating it from the odontoblasts consisted of $P^{32}$-free dentin formed between the first and fourth day. There was little decrease in the intensity of the reaction line with time, presumably because the crystals of dentin formed soon after injection, were labeled throughout, while only the phosphate groups of their surface were

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* This work was assisted by grants from the National Research Council of Canada to Dr. L. F. Bélanger, and from the Associate Committee on Dental Research of the Council to Dr. C. P. Leblond.

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5. Hevesy, G., *Radioactive indicators*, Interscience Publishers, Inc., New York, 1946.

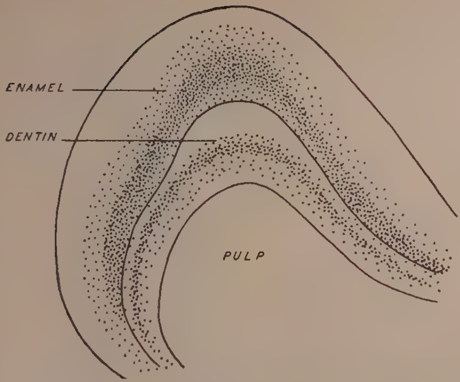

Fig. 1.

Inverted autograph of a section of a molar cusp of an 8-day-old hamster injected with $P^{32}$ 4 days previously. The dentin shows an autographic reaction as a rather narrow dark line of granules in its mid-portion. This line moved from the inner edge of the dentin (1 day after injection) to its present position. The layer of enamel shows a reaction as a broad, dark line of granules at a distance from the ameloblasts and the dentino-enamel junction.

liable to lose their label by exchange. It was concluded that dentin grows by continuous apposition of layers of insoluble phosphate in the area close to the odontoblasts,—a process similar to the formation of periosteal and endosteal bone(6).

The deposition of phosphate in young enamel occurred at a rather considerable distance from the ameloblasts, the reaction being spread over a much broader line than that of the dentin (Fig. 1). The distance between the reaction line and the ameloblasts appeared to increase with time. The reaction did not significantly decrease in intensity with time and, therefore, was attributed to newly formed enamel crystals. Parallel studies with the von Kossa stain for calcium phosphate revealed a progressive increase in calcification from the outer (ameloblastic) limit to the inner (dentinal) limit of the enamel; there was an area of maximum calcification near the apex of the dentino-enamel junction. The radio-phosphate was deposited with a similar gradient, which, however, did not extend as far inwards as the area of maximum calcification. These findings were in agreement with the concept that the formation of enamel is due to the elaboration by the ameloblasts of an organic matrix, which is mineralized much later(7).

The results gave no information as to why the crystals of dentin and enamel form in the indicated areas.

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6. Leblond, C. P., Wilkinson, G. W., Bélanger, L. P., and Robichon, J., *Am. J. Anat.*, 1950, in press

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## Synthesis of Sulfur Amino Acids from Inorganic Sulfate by Ruminants.*
## (17693)

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The conversion of nitrogen of simple compounds in the diet to body protein of the ruminant, probably through the mediation of the micro-organisms of the paunch, is now generally accepted(1,2). However, there have

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* These investigations have been conducted under authorization from the U. S. Atomic Energy Commission, serial No. 4022 and 4051, Oak Ridge National Laboratory, Oak Ridge, Tenn. Aided in part by a grant from the Borden Company, New York City.

1. Mitchell, H. H., and Hamilton, T. S., The Biochemistry of the Amino Acids., Chemical Catalogue Co., New York, 1929.

TABLE I.
Radioactivity of Milk Samples After Feeding $Na_2S^{35}O_4$.

| Day No. | Time of collection | Activity in 1 ml milk in counts/min. | Activity in total collection counts/min., ×1000 |
|---|---|---|---|
| 1 | P.M. | 21 | 63 |
| 2 | A.M. | 73 | 370 |
| 2 | P.M. | 92 | 204 |
| 3 | A.M. | 82 | 317 |
| 3 | P.M. | 54 | 118 |
| 4 | A.M. | 50 | 105 |
| 4 | P.M. | 44 | 84 |
| 5 | A.M. | 45 | 145 |
| 5 | P.M. | 29 | 71 |
| 6 | A.M. | 34 | 107 |
| 6 | P.M. | 28 | 54 |
| 7 | A.M. | 27 | 62 |
| 7 | P.M. | 21 | 39 |
| 8 | A.M. | 22 | 54 |
| 8 | P.M. | 17 | 30 |
| 9 | A.M. | 14 | 45 |
| 10 | ” | 18 | 42 |
| 11 | ” | 24 | 56 |
| 12 | ” | 14 | 31 |
| 13 | ” | 11 | 21 |
| 20 | P.M. | 10 | 14 |
| 27 | A.M. | 20 | 27 |

not been any reports in the literature, to our knowledge, pertaining to the conversion of sulfate sulfur to cystine and methionine sulfur of tissue proteins. The experiments described below report evidence of this conversion.

*Experimental.* Approximately 9 millicuries of $S^{35}$ in the form of $Na_2S^{35}O_4$ were fed in divided doses to a Holstein cow weighing approximately 1300 lb over the course of 8 hours. The milk was collected and the activity was measured on an aliquot as follows: 1 ml of each sample was pipetted into a planchet and evaporated under a "heat lamp" to dryness, then counted in a G-M counter with less than 2 mg mica window counter of 5 sq cm area. In all cases, the background counts were made before and after each determination of activity, and subtracted from the results obtained on the samples. The results in Table I are expressed in counts per minute of the total quantity of milk collected for each sample. A few casual samples of urine were collected, and these had the following activity in counts per minute per ml: sample I, collected 2 hours after feeding the radioactive sulfate, 463; sample II, collected 8 hours after feeding, 973; sample III, collected 5 days after feeding, 194. The proteins were isolated from milk collected on the following days: 2nd morning; 3rd morning; 11th morning; 12th morning. Ten grams of trichloracetic acid dissolved in 10 ml of water were added to 100 ml of milk from each of these days. The precipitates were allowed to form in the cold and they were then removed by centrifugation. The protein precipitates were washed very thoroughly five times with cold 2% solution of trichloracetic acid. The filtrates and washings were combined, concentrated to 100 ml and the activity was determined. The acid-washed protein precipitates were extracted 3 times with hot acetone and once with ether. The proteins were dried under a "heat

TABLE II.
Radioactivity of Milk Proteins and Protein-free Filtrates Counts Per Minute.

| Day No. | Activity in milk protein from 100 ml of milk | Activity in protein-free filtrate from 100 ml of milk |
|---|---|---|
| 2 | 6200 | 5800 |
| 3 | 7200 | 3100 |
| 11 | 710 | <100 |
| 12 | 860 | <100 |

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2. E. I. du Pont de Nemours and Co., Inc., Review of Nutritional Research on Urea as a Supplementary Source of Protein for Ruminants, Wilmington, Del., 1949.

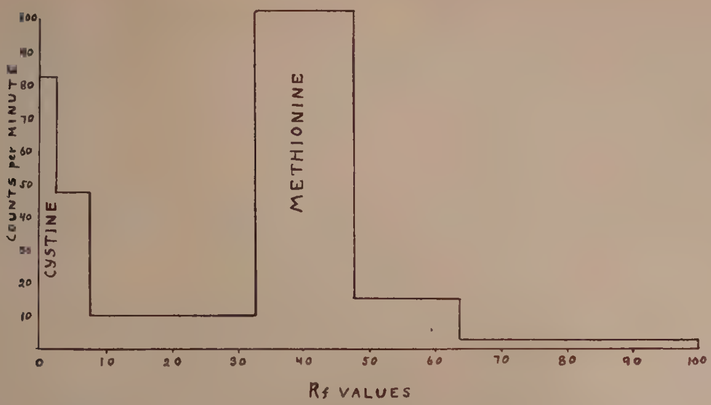

FIG. 1.
Radio paper chromatogram of 100 mg milk proteins hydrolysate.

lamp". The radioactivity of the filtrates and the proteins is recorded in Table II. One hundred milligrams of the sample of milk proteins which show the highest activity (third morning) were hydrolyzed with 10 ml of 6 N HCl under reflux for 24 hours. The hydrolysate was decolorized with 100 mg of carbon and the excess of HCl was removed by evaporation to dryness at 35° *in vacuo* and repeating the process on addition of water 3 times. The dry residue was then taken up in 5 ml of water. One-hundredth ml aliquots of the hydrolysate, 12 mm apart, were put on 5 sheets (18 x 21 inches) of S. and S. No. 598 filter paper until the entire 5 ml of the hydrolysate were applied. Cystine, methionine, and $Na_2S^{35}O_4$ solutions were also applied to each sheet for control purposes. The chromatograms were developed with water-saturated N butanol-acetic acid (10 $^v/_v$ for 24 hours (3). Control strips cut from the sheets indicated that the sulfate does not move from the point of application with this solvent[†] Cystine had an Rf of 0.04 (0.03-0.05) and methionine an Rf value of 0.41 (0.39-0.43). In view of these findings, the sheets were cut as follows: Band I, Rf 0.00-0.03; Band 2, Rf 0.03-0.05; Band 3, Rf 0.08-0.33; Band 4, Rf 0.33-0.47; Band 5, Rf 0.47-0.64; and Band 6, Rf 0.64-1.00. The analogous bands from each of the 5 sheets were thoroughly extracted with hot water, the extracts were concentrated to dryness, and the activity of each extract was determined as described above. The results are summarized in Fig. 1.

*Results.* Our data indicate that sulfate sulfur is converted in appreciable quantities to cystine and methionine sulfur when fed to a ruminant. It has been previously shown(4) that even non-ruminants are able to exchange a small quantity of sodium sulfide sulfur for the cystine sulfur of their tissue proteins. However, there are no experiments which show that sulfate sulfur will function in this manner. As far as we are aware, no evidence has been presented which indicates the synthesis of methionine from inorganic sulfate, although this is known to be true in the case of many micro-organisms. Whether the synthesis of cystine and methionine from inorganic sulfate proceeded via two independent paths or whether the cystine originated from the synthesized methionine or *vice versa* in our cow is not possible to assess from the data at hand.

*Summary.* Radioactive sodium sulfate was fed to a cow, milk was collected for several days, and the proteins were isolated. The

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3. Block, R. J., and Bolling, D., The Amino Acid Composition of Proteins and Foods, 2nd Edition, C. C. Thomas, Springfield, Ill., 1950.

[†] The $BaSO_4$ precipitate from the hydrolysate of 422 mg of milk proteins (third morning) gave only 30 counts per minute.

4. Dziewiatkowski, D. D., *J. Biol. Chem.*, 1946, v164, 165.

proteins showed appreciable radioactivity. From the hydrolysate of the radioactive proteins cystine and methionine were separated on chromatograms, and eluted. Both cystine and methionine contained radioactive sulfur in appreciable amounts.

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## Relative Resistance of Newborn Mice to Poliomyelitis Virus.* (17694)

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The present study was begun with the hope that propagation of the Lansing strain of poliomyelitis virus in newborn mice might yield material with higher virus titers. It appeared probable that this might be achieved, because repeated observations in this laboratory(1) indicated that when the mouse-adapted Hawaii dengue virus, which also produces flaccid paralysis in intracerebrally inoculated mice, is injected in suckling mice less than one week old, the infected brain suspensions usually have virus titers, which are approximately 100 times greater than those obtained from brains of mice inoculated at 2 weeks of age or older. However, a preliminary test carried out with the Lansing virus suggested that the newborn mice may be more resistant that the older animals. Since this observation was not in accord with the recorded properties of all other neurotropic viruses in mice(2,3,4), as systematic study was carried out on larger numbers of animals to elucidate this unexpected behavior of poliomyelitis virus.

*Methods.* The albino mice used in these tests were obtained from a Cincinnati dealer, who could not be certain of their genetic origin or composition except that they were probably "Swiss" mice. Pregnant females obtained from the dealer several days before expected delivery of young, were placed in separate boxes and the birth of new litters was noted twice daily. Mice were not used on the day of birth. All the tests were carried out during the months of June, July and August, 1948, when the temperature in the animal quarters was not infrequently 90 to 100°F. Following inoculation the suckling mice were returned to their mothers, and each family was observed separately. The virus consisted of a large lot of 10% centrifuged mouse brain suspension in saline, aliquots of which were distributed in sealed glass ampules which were maintained in the frozen state in a "dry ice" storage box; it represented the 203rd mouse brain passage and had an $LD_{50}$ of $1 \times 10^{-3.7}$ for the 0.03 ml dose. Since only 0.01 ml was inoculated intracerebrally in the suckling mice, the dose for the older mice, used for control in each test, was also 0.01 ml. The inoculations were carried out without anaesthesia.

*Results.* When 0.01 ml of the $10^{-1}$ virus suspension, containing approximately 170 $LD_{50}$ (based on titration in mature mice using 0.03 ml doses), was inoculated intracerebrally in mice of different ages, the significant difference between the newborn and older mice was in the length of the incubation period (Table I) and in the survival time after onset of paralysis (Table II). With regard to prolongation of the incubation period, there was no apparent difference between suckling mice tested 1, 2 or 3 days after birth, it was still definite but progressively less marked in those tested at 4 to 7 days, while at 15 days after

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* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

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2. Olitsky, P. K., Sabin, A. B., and Cox, H. R., *J. Exp. Med.*, 1936, v64, 723.

3. Casals, J., *J. Exp. Med.*, 1940, v72, 445.

4. Lennette, E. H., and Koprowski, H., *J. Immunol.*, 1944, v49, 175.

TABLE I.
Incubation Periods in Mice of Different Ages Inoculated with Lansing Virus.

| Age of mice inoculated days | No. of mice in group | % paralyzed or dead on indicated day after intracerebral inoculation of 0.01 ml of 10-1 virus | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | % remained well |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 31 | 32 | 33 | 34 | 35 | 44 | |
| 1 | 52 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 13 | 19 | 35 | 38 | 50 | 63 | 65 | 75 | 81 | 85 | 85 | 87 | 87 | 87 | 87 | 87 | 88 | 90 | — | — | 92 | — | — | — | 7.7 |
| 2 | 87 | 0 | 0 | 0 | 0 | 3 | 5 | 8 | 11 | 16 | 23 | 41 | 53 | 60 | 68 | 72 | 75 | 78 | 79 | 79 | 84 | 86 | — | — | — | — | — | — | 87 | 89 | — | — | 11.5 |
| 3 | 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 6 | 12 | 24 | 46 | 64 | 72 | 78 | 82 | 90 | 90 | 92 | — | — | — | — | — | — | 94 | — | — | — | — | 96 | 4.0 |
| 4 | 39 | 0 | 0 | 8 | 8 | 10 | 10 | 15 | 21 | 26 | 36 | 59 | 72 | 82 | 87 | 90 | 90 | 90 | 92 | — | — | — | — | — | — | — | — | — | — | — | — | — | 7.7 |
| 6 and 7 | 40 | 0 | 0 | 7 | 7 | 10 | 15 | 27 | 40 | 45 | 65 | 72 | 80 | 80 | 90 | 90 | 90 | 92 | 95 | — | — | — | — | — | — | — | — | — | — | — | — | — | 5.0 |
| 15 (suckling) | 38 | 0 | 0 | 16 | 29 | 42 | 55 | 66 | 74 | 79 | 84 | 87 | 89 | 92 | 95 | — | — | — | — | — | — | — | — | 97 | — | — | — | — | — | — | — | — | 2.6 |
| 15 (weaned) (litter mates) | 28 | 0 | 0 | 25 | 43 | 57 | 75 | 82 | 86 | 89 | — | — | 93 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 7.1 |
| 21-35 | 157 | 0 | 3 | 16 | 32 | 48 | 54 | 64 | 72 | 78 | 85 | 87 | 90 | 90 | 92 | 94 | 94 | 95 | — | — | — | — | — | 96 | — | — | — | 97 | — | — | — | — | 2.6 |

TABLE II.
Survival Time of Mice Developing Paralysis During First 14 Days After Birth.

| Age at time of inoculation days | Time after birth paralysis appeared days | No. mice in group | % of mice surviving indicated No. of days after paralysis first observed | | | | | | | | | Avg survival time,* days, |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
| 1-6 | 14 or less | 71 | 18 | 21 | 20 | 14 | 10 | 7 | 6 | 1 | 3 | 3.44 |
| 1-6 | 15 or more | 92 | 64 | 24 | 8 | 3 | 1 | 0 | 0 | 0 | 0 | 1.53 |
| 21-35 | 23 " " | 100 | 76 | 20 | 0 | 2 | 1 | 1 | 0 | 0 | 0 | 1.35 |

* Only mice in whom paralysis was observed prior to death are included in this analysis. This does not affect the average survival time of the suckling mice paralyzed within 14 days after birth, because only 2 of the total number were found dead without previously exhibiting paralysis, but exaggerates the average survival time of the others, because in approximately 20 to 30% the interval between onset of paralysis or other CNS signs and death is too short to be observed when the examinations are made only once a day.

birth the distribution of incubation periods among 38 suckling mice was practically identical with that found among 157 weaned mice, 3 to 5 weeks of age (Table I). To test the effect of premature weaning, 28 mice were removed from their mothers at 15 days after birth and tested simultaneously with their litter mates which were left with their mothers. The prematurely weaned mice received bread and water until they were big enough to chew pellets and reach the water bottles. The mice weaned at 15 days of age succumbed more rapidly than their suckling litter mates and the older weaned mice (Table I), but the number used in the test is not large enough

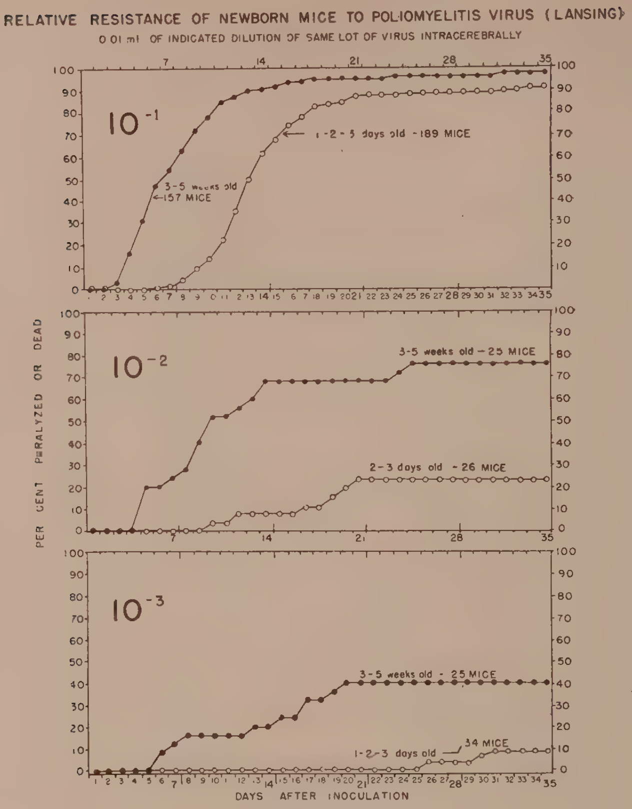

FIG. 1.

to permit significant deductions. With regard to prolongation of survival time after onset of paralysis, it was noted that the determining factor was not the age of the mouse at the time of inoculation but rather its age at the time paralysis first appeared. Inspection of the protocols indicated that the critical period was around 14 days after birth. Mice becoming paralyzed during the first 14 days of life generally (61% of 71 mice) survived 3 or more days, and survival times of 5 to 9 days were not unusual (27%). However, when the incubation periods were prolonged beyond 15 days of age the survival time pattern among the remaining litter mates approached that observed among the older weaned animals (Table II).

When the inoculum was reduced to 0.01 ml of the $10^{-2}$ and $10^{-3}$ dilutions of virus, the difference was not only in the prolongation of the incubation period but also in the larger number of survivors without paralysis among the 1- to 3-day-old suckling mice Fig. 1). These mice were observed for 70 days after inoculation without any change in the results shown in Fig. 1 for the first 35 days. Calculation of the $LD_{50}$ on the basis of the data for the 0.01 ml dose, shown in Fig. 1, yields a value of $10^{-1.67}$ for the 1- to 3-day-old mice and a minimum estimated value of 1 x $10^{-2.64}$ for the 3- to 5-week-old mice; it should be recalled, however, that a complete titration of this virus preparation in older mice, including the $10^{-4}$ and $10^{-5}$ doses, yielded an $LD_{50}$ of 1 x $10^{-3.7}$ for the 0.03 ml dose, with an estimated $LD_{50}$ of 1 x $10^{-3.2}$ for the 0.01 ml dose. Thus, the minimal virus inoculum required to produce the disease was 10 to 30

TABLE III.
Level of Virus Multiplication in Suckling and Older Mice at Onset of Paralysis.

| | | | Titration of suspension of spinal cords and brain stems | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | No. of mice succumbed in 35 days after inoculation with indicated dilution | | | | | |
| Age at inoculation, days | Days after inoculation paralyzed | No. mice | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $LD_{50}$ |
| 2 | 11 | 4 | 10/10 | 10/10 | 7/10 | 1/10 | 0/10 | 3.3 |
| 28-35 | 10-11 | 4 | 10/10 | 8/10 | 6/10 | 3/10 | 1/10 | 3.3 |

TABLE IV.
Effect of Intracerebral Injection of Lansing Virus on Suckling and Mature Mice of "C-57 Black" Strain 0.01 ml of $10^{-1}$ Virus.

| | 3 days old<br>Days after inoculation | | 5 days old<br>Days after inoculation | | 4-6 weeks old<br>Days after inoculation | |
|---|---|---|---|---|---|---|
| Mouse No. | Paralyzed | Dead | Paralyzed | Dead | Paralyzed | Dead |
| 1 | 8 | 11 | 3 | 11 | 4 | 6 |
| 2 | 8 | 13 | 3 | 11 | 5 | 6 |
| 3 | 9 | 14 | 3 | 11 | — | 6 |
| 4 | — | 10 | 9 | 12 | 6 | 7 |
| 5 | 10 | 12 | — | 12 | — | 7 |
| 6 | 10 | 13 | 16 | 18 | 7 | 9 |
| 7 | 10 | 14 | | | — | 8 |
| 8 | 11 | 15 | | | 9 | 10 |
| 9 | 14 | 16 | | | 9 | 11 |
| 10 | | | | | 10 | 11 |
| 11 | | | | | 11 | 12 |
| 12 | | | | | 11 | 12 |
| 13 | | | | | — | 12 |
| 14 | | | | | 13 | 14 |
| 15 | | | | | 16 | 17 |
| 16 | | | | | 42 | 43 |
| 17 | | | | | — | survived 60 |
| Mean survival time after onset of paralysis | 3.5 days | | 5.8 days | | 1.2 days | |

times greater for the 1 to 3-day than for the 21- to 35-day-old mice.

*Level of Virus Multiplication at Onset of Paralysis in Suckling and Older Mice.* The available data did not indicate whether the extent to which the virus had to multiply for the production of paralysis was greater or smaller in sucklings than in older mice. Accordingly, 4 mice, which were inoculated with 0.01 ml of $10^{-1}$ virus at 2 days of age, were sacrificed at the first appearance of paralysis 13 days after birth, and a pool of their spinal cords and brain stems was titered for virus content. On the same day were also sacrificed 4 moribund mice, which were 4 to 5 weeks old at the time of inoculation with the same dose of virus; 3 of these also had an incubation period of 11 days and one of 10 days, and a pool of their spinal cords and brain stems was titered simultaneously with that obtained from the suckling mice. The results shown in Table III indicate that at onset of paralysis, the level of virus multiplication was the same in suckling and older mice. Thus the greater survival time after onset of paralysis would appear to depend on factors other than the level of viral multiplication in the spinal cord and brain stem.

*Behavior of Lansing Virus in Sucklings of Other Genetic Strains of Mice.* Small numbers of "W-Swiss" suckling mice, derived from mothers bred in this laboratory or from "CFW" pregnant females sent by Carworth Farms, exhibited the same type of resistance as the albino mice used in the tests described above. The results obtained with "C-57

TABLE V.
Immunity of Mice Surviving Previous Intracerebral Inoculation of Lansing Virus.
Challenge Dose = 100 $LD_{50}$ Intracerebrally.

| Previous inoculation | No. tested | % paralyzed or dead on indicated day | | | | % resistant |
|---|---|---|---|---|---|---|
| | | 7 | 14 | 21 | 32 | |
| During 1st week of life | 24 | 62.5 | 62.5 | 75.0 | 75.0 | 25.0 |
| After 2nd " " " | 12 | 33.3 | 33.3 | 41.7 | 50.0 | 50.0 |
| Total | 36 | 52.8 | 52.8 | 63.9 | 66.7 | 33.3 |
| None—mothers of above | 43 | 60.0 | 81.4 | 88.4 | 90.7 | 9.3 |
| " —new "old" controls | 24 | 71.0 | 96.0 | 100.0 | — | 0 |

Black" mice, bred in this laboratory from Jackson Memorial stock, are shown in Table IV. Although the number of mice is too small for a significant comparison of incubation periods, the difference in survival time after onset of paralysis is very marked and similar to that observed in the suckling and older albino mice.

*Immunity of Mice Unaffected by Previous Intracerebral Inoculation.* The purpose of this test was to determine whether the mice which were unaffected by an intracerebral inoculation of $10^{-1}$ Lansing virus during the first week of life may have experienced an inapparent infection leading to resistance to further inoculations of virus. Twenty-four such mice were reinoculated with approximately 100 $LD_{50}$ of virus 35 to 80 days after the initial injection. Twelve unaffected, surviving mice, which received their first inoculation at 15 to 35 days of age, were reinoculated as a check on the results which might be obtained with the first group. The mothers of the above survivors and older mice, weighing approximately 30 g and not previously exposed to inoculated animals, served as controls for the activity of the virus in mice of more advanced age. The results shown in Table V indicate that 75% of the 24 mice, which resisted a primary inoculation during the first week of life, succumbed to the second inoculation of virus, strongly suggesting that the first inoculation was not followed by inapparent infection. The survival of 25% of these mice and of 50% of the 12 which had the primary inoculation at 15 to 35 days of age, probably is significant by comparison with the previously uninoculated controls. However, it may only mean that the survivors represent a selected group, which regardless of age require a larger inoculum to initiate infection. Since there was no evidence of inapparent infection in the newborn mice, it may be justifiable to conclude that larger amounts of poliomyelitis virus are required to initiate multiplication in their tissues than in those of adult mice.

*Discussion.* The present data for poliomyelitis virus may be compared with the intracerebral titrations of 9 other neurotropic viruses carried out simultaneously in 3-day and 28-day-old mice by Lennette and Koprowski(4). They found that the minimal amount of virus which had to be inoculated to produce the clinically apparent disease was either the same or smaller for the 3-day than for the 28-day-old mice. Casals(3) showed that approximately 100 times more rabies virus had to be injected intracerebrally to produce the disease in full grown than in 7-day-old mice. Incubation periods have also been found to be shorter in very young mice intracerebrally inoculated with other neurotropic viruses(2). Thus far, poliomyelitis virus would appear to be unique in its capacity to multiply more readily and rapidly in the nervous system of older mice. This would suggest that multiplication of poliomyelitis virus in mice may require some factor or metabolic pathway which is different from the other neurotropic viruses studied thus far. Pinto(5) found that while peripherally inoculated Lansing virus was equally without effect in suckling and older mice, it invaded the

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5. Pinto, M. R., *Am. J. Hyg.*, 1948, v48, 361.

central nervous system more readily in suckling than in older cotton rats. Although it is possible that the virus may behave differently in newborn cotton rats and mice, it is also possible that invasiveness from peripheral sites and multiplication within the central nervous system may depend on different mechanisms. It is perhaps noteworthy, in this respect, that it is generally agreed that poliomyelitis infection in human beings more commonly produces severe clinical disease in older individuals than in very young children, past the age of possible protection by placentally transmitted antibody.

*Summary.* The Lansing strain of poliomyelitis virus, unlike other neurotropic viruses in mice, produced the disease more readily and rapidly in mature mice than in newborn animals. The relative resistance of the newborn manifested itself by prolonged incubation periods and survival times after onset of paralysis in those inoculated with larger amounts of virus (170 $LD_{50}$), and by a failure to develop the disease following the intracerebral inoculation of smaller amounts (2-17 $LD_{50}$). The prolongation in incubation period was most marked in mice inoculated at 1, 2 and 3 days of age, was still definite but progressively less marked in those tested at 4 to 7 days of age, while suckling mice inoculated at 15 days of age exhibited incubation periods almost identical in their distribution with those of weaned, 21 to 35-day-old mice. The amount of intracerebrally inoculated virus required to initiate infection in newborn mice 1 to 3 days of age was 10 to 30 times greater than in 21- to 35-day-old mice. Although the incubation period was markedly prolonged in the newborn mice, the level of virus multiplication attained at the onset of paralysis was the same in the suckling as in the older mice. Mice becoming paralyzed during the first 14 days of life survived much longer (61% for 3 to 9 days) than the litter mates whose incubation periods were prolonged beyond 15 days of age (12% for 3 to 5 days). There was no evidence of inapparent infection in mice inoculated during the first week of life or subsequently, since the majority of surviving mice succumbed to a second inoculation of virus 35 to 80 days later.

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## Conditioning, Through Stress, of the Action of Corticoids on Lymphoid Organs.* (17695)

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*In vitro* experiments concerning the action of corticoids on lymphocytes are at present inconclusive. While numerous investigators have suggested that gluco-corticoids have cytotoxic effects on lymphoid cells *in vitro* (1,2,3,7), other workers have found that total cortical extracts did not show this lympholytic action(4,5). The recent experiments of Hechter and Johnson(6) can perhaps explain the different effects of corticoids *in vitro* and *in vivo* on lymphoid cells. These investigators have found that total cortical extract is an effective lympholytic agent *in vitro* only in the presence of homogenates of lymphoid tis-

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* This work has been subsidized by a grant from the National Cancer Institute of Canada.

1. Heilman, D. H., *Proc. Staff Meet. Mayo Clin.*, 1945, v20, 310.

2. Heilman, D. H., *Proc. Staff Meet. Mayo Clin.*, 1945, v20, 318.

3. Hechter, O., and Stone, D., *Fed. Proc.*, 1948, v7, 52.

4. Dougherty, T. F., and White, A., *Am. J. Anat.*, 1945, v77, 81.

5. Robertson, J. S., *Nature*, 1948, v161, 814.

6. Hechter, O., and Johnson, S., *Endocrinology*, 1949, v45, 351.

7. Schrek, R., *Endocrinology*, 1949, v45, 317.

sue and their results suggest that the lymphoid tissue co-factor is perhaps enzymatic. The caryoclastic action of gluco-corticoids and stress on lymphoid organs *in vivo,* is well known. However, while Selye(8,9) has shown that adrenalectomy prevents the thymus caryoclasia caused by stress, we have obtained some preliminary data suggesting that even in the absence of the adrenals slight caryoclasia can occur in the lymph nodes. It seems therefore that the effects of stress on the lymphoid tissue cannot be totally explained by the secretion of corticoids. Consequently, we have performed the following experiment to obtain additional information concerning this question.

We have studied the influence of 3 stress-producing agents, cold, forced muscular exercise and 40% formaldehyde injections on the action of corticoids in the adrenalectomized rat.

The experimental animals, male piebald rats weighing 150-180 g were subdivided into 4 groups and treated as follows:

(1) The first group consisted of 9 rats, each of which received a single 0.2 cc injection of cortical extract (Upjohn lipo-adrenal cortex) 24 hours following adrenalectomy.

(2) The rats of the second group were similarly treated with 0.2 cc of the cortical extract 24 hours following adrenalectomy. Immediately after the injection, 6 of the animals were placed in the cold room (0°-5°C) for a period of 6 hours; 6 were subjected to forced exercise in a drum for the same period of time and the remaining 6 were given, within a 6 hour period, two 0.2 cc injections of 40% formaldehyde.

(3) A third group of 18 rats which likewise had undergone adrenalectomy 24 hours previously was subdivided into 3 equal groups and submitted to the same stresses as in (2) above but without the cortical extract pretreatment.

(4) A fourth group of 18 normal rats was similarly subdivided into 3 groups of 6 rats each and submitted to the same stresses also without hormone treatment.

All groups were sacrificed by chloroform inhalation 6 hours following either exposure to stress or injection of cortical extract. At autopsy, the thymus as well as a portion of the intestine with its Peyers patches were removed from the rats of each group. Alcohol-formal-acetic acid was used as the fixative and staining was done either with hematoxylin-eosin or with Feulgen's stain.

*Results.* In the first group, it was found that 6 hours after the injection of the cortical extract, the lympholytic effect was hardly manifest. Areas of pyknosis were few and,

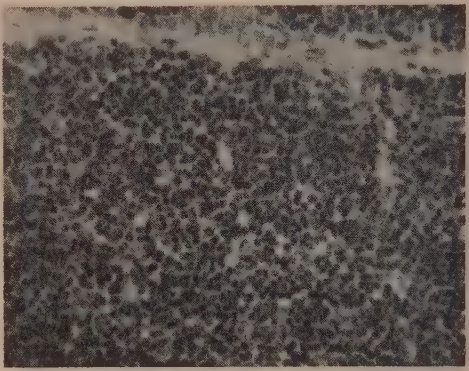

FIG. 1.
Thymus of an adrenalectomized rat treated with 0.2 ml of lipoadrenal cortex extract (Upjohn). The absence of caryoclasia of the thymocytes can be clearly seen.

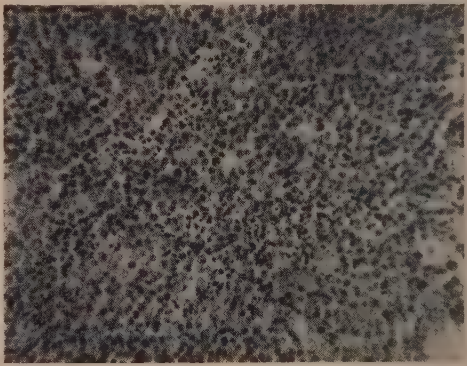

FIG. 2.
Similar section of thymus from an adrenalectomized rat treated with the same dose of cortical extract but exposed to muscular exercise. Note the development of confluent areas of caryoclasia of thymocytes.

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8. Selye, H., *Brit. J. exp. Path.*, 1936, v17, 234.
9. Seyle, H., *Endocrinology*, 1937, v21, 169.

in most of the animals, the thymus and Peyers' patches were entirely normal in appearance.

In the second group where the effect of stress was added to the action of the same dose of corticoids, extensive caryoclasia had taken place in the thymus and in the germinal centers of the Peyers' patches. In both tissues, massive and often confluent areas of pyknosis could be observed, their aspect remaining the same despite the type of stress to which the animals had been subjected.

In the third group, thymus caryoclasia was inhibited by the adrenalectomy, yet a very few areas of pyknosis could be observed in the Peyers' patches.

In the fourth group the classical picture of caryoclasia was observed in both lymphoid organs of the normal rats subjected to stress. It was noted however, that the caryoclasia was often less pronounced than it was in the second group of animals, particularly in those subjected to forced muscular exercise.

*Comments.* These experiments show that the influence of corticoids on the lymphoid system may be conditioned by non-specific stress. They militate in favor of the view that these hormones act on the lymphoid cells by some indirect means as well as imply that some conditioning factors related to the stress (enzymes?) influence this action.

*Summary.* The combined action of a small dose of cortical extract and non-specific stress of moderate intensity has a well marked caryoclastic effect on the lymphoid system of adrenalectomized rats, whilst the same dose of extract given to adrenalectomized, non-stressed rats, produces a negligible effect.

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## Demethylation of $C^{14}$-Labelled Codeine in the Rat.* (17696)

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The demethylation of codeine has been postulated by Sanfillipo(1) on the basis of the presence of typical morphine withdrawal symptoms in dogs after the prolonged administration of codeine. More direct evidence was obtained by Bernheim's *in vitro* studies which showed that codeine after incubation with rat liver slices gave rise to a phenolic-OH compound, presumably morphine (2). Since Bernheim has also shown that morphine itself is oxidized at an appreciable rate, the extent to which demethylation occurs is presumably greater than that indicated by quantitative tests for residual morphine. Our experiments with radiocodeine (codeine*) now establish by direct evidence that codeine is demethylated in rats at the 3-position. The present studies were initiated by the observation that $C^{14}O_2$ rapidly appeared in the expired air of rats after administration of codeine labelled with $C^{14}$ in the methoxy group. The present data include measurements of $C^{14}O_2$ output *in vivo* and analyses of tissues for $C^{14}O_2$-$HC^{14}O_3^-$concentrations in addition to studies on the ability of various tissues to demethylate codeine* *in vitro*.

*Methods.* The animals used were adult, male and female albino rats of the Slonaker-Wistar strain maintained on a diet of Friskies dog food and tap water. The codeine* was synthesized by Chang, *et al.*(3), and had a specific activity of 0.9 microcuries/mg. The

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* Supported, in part, by a grant from the National Institutes of Health, Bethesda, Md.

1. Sanfillipo, G., *Boll. Soc. Ital. Biol. Sper.*, 1948, v24, 723.

2. Bernheim, F., and Bernheim, M. L. C., *J. Pharm. and Exp. Therap.*, 1944, v81, 374.

3. Chang, F. N-H., Oneto, J. F., Sah, P. P. T., Tolbert, B. M., and Rapoport, H., to be published in *J. Org. Chem.*

$C^{14}$ was determined by the method of Dauben, *et al.*(4).

*In vivo studies.* The animals received a subcutaneous injection of 40 mg codeine* per kilo into the left leg and were placed in a glass metabolism cage. Expired $CO_2$ was collected in NaOH towers for periods of 1-31 hours and analyzed for $C^{14}$. For tissue analyses of radioactive $CO_2$-$HCO_3^-$, ether was administered to one rat 2 hours after codeine* administration, and a blood sample was obtained from the abdominal aorta. After death by exsanguination, the organs were removed and immediately placed in Potter homogenizers containing 0.5 N NaOH. Aliquots of the homogenate were acidified in the apparatus used for the *in vitro* studies, and the $CO_2$ collected for the determination of $C^{14}$. The residue after acidification was dried at 60° and combusted for the determination of the remaining $C^{14}$ by the method of Van Slyke and Folch(5) as modified by Skipper, *et al.* (6).

*In vitro studies.* Rats were killed by decapitation, and the required organs were removed rapidly and placed in a cold box(7). Liver and kidney slices were prepared with a Martin slicer(8); lungs, spleen, brain, intestine and muscle (diaphragm) were cut into strips with a scalpel. Blood was drawn from the abdominal aorta into a heparinized syringe. Incubation of all tissues took place in an apparatus of the type described by Entenman for the wet oxidation of tissues(9).

The tissue slices were suspended in an Erlenmeyer flask containing 20 ml of Krebs-Ringer phosphate buffer(10) per g of tissue, with 0.2% glucose and 630 μg codeine* added. In the case of the experiments with blood, the Krebs-Ringer solution was omitted, the codeine* being added directly. Oxygen saturated with water vapor was admitted throughout the experiment, and the $CO_2$ produced was absorbed in a sodium hydroxide tower. The incubation flask was immersed in a water bath at 37°. At the end of 2 hours 2 ml of 1 N HCl were added to the flask in order to stop the action and decompose any bicarbonate formed. In the liver slice experiments a control flask in which HCl was added at the beginning of the experiment was set up under the same conditions. At the end of the experiment the incubation fluid was separated from the tissue slices by filtering through glass wool. The fluid was analyzed for $C^{14}$ as well as for basic amines by the methyl orange technique of Brodie(11). The latter method was appraised for specificity by comparing codeine

TABLE I.
*In vivo* Formation of $C^{14}O_2$ from Codeine*.

| Rat No. | Time after inj. | Mg codeine* appearing as $CO_2$ | % dose inj. |
|---|---|---|---|
| 1 | 2 hr | 2.05 | 22.2 |
| 3 | ,, ,, | 1.14 | 19.1 |
| 4 | ,, ,, | 1.12 | 14.1 |

* Labelled radioactive compound.

TABLE II.
Tissue Distribution of $C^{14}O_2$ and $HC^{14}O_3^-$ Resulting from Demethylation of Radio-codeine After Codeine* Administration.

| Tissue | % dose inj. | Ratio $\frac{C^{14} \text{ as } CO_2\text{-}HCO_3^-}{\text{Total tissue } C^{14}}$ |
|---|---|---|
| Liver | .23 | .034 |
| Blood | .20 | .091 |
| Intestine | .13 | .046 |
| Fat | .06 | — |
| Urine | .04 | .012 |
| Kidneys | .03 | .038 |
| Stomach | .03 | .011 |
| Brain | .02 | .067 |
| Spleen | .02 | .043 |
| Ovaries | .02 | .019 |
| Lungs | .01 | .028 |
| Heart | .003 | .031 |

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4. Dauben, W. G., Reid, J. C., and Yankwich, P. E., *Anal. Chem.*, 1947, v19, 828.

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7. Fuhrman, F. A., and Field, J., 2nd, *J. Biol. Chem.*, 1944, v153, 515.

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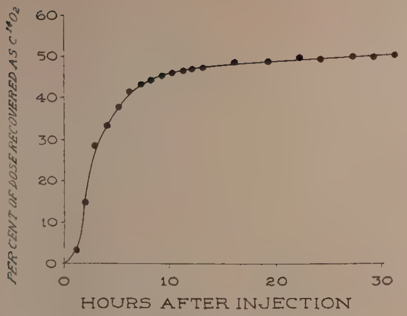

FIG. 1.
Recovery of $C^{14}O_2$ in expired air following injection of methoxy-labelled codeine.

distribution ratios between benzene and phosphate buffers of pH values 7.35, 7.12 and 6.72 respectively.

*Results. In vivo studies.* Approximately one-fifth of the radioactivity administered as a subcutaneous injection of 40 mg/kg codeine* appeared as $C^{14}O_2$ in the expired air within 2 hours (Table I). A small additional amount, less than 1% of the injected dose, was found distributed as $C^{14}O_2$-$HC^{14}O_3^-$ in the tissues at this time. Furthermore, analyses of the individual tissues indicate that the contribution of such radioactivity to the total activity of the tissue is minor (Table II). Thus, even in the case of the blood the $C^{14}$ due to $C^{14}O_2$-$HC^{14}O_3^-$ is less than one-tenth of the total $C^{14}$ content of that tissue. A detailed analysis of $C^{14}O_2$ excretion over a 31 hour period is presented in Fig. 1. A total of 51% of the radioactivity administered (as codeine*) was excreted during this period, the greatest part of this coming off during the first 6 hours. In this animal, peak $C^{14}O_2$ production occurred 3 hours after the subcutaneous injection.

*In vitro studies.* No demethylation was observed in our experiments (2 each) with blood and slices of lung, spleen, brain, intestine and the control liver experiment. Although both kidney and muscle exhibited a definite but limited capacity for demethylation, by far the most important site for this process was the liver (Table III).

Analysis of residual codeine (after incubation with liver slices) by the methyl orange technique showed good agreement with results obtained by analyzing for $C^{14}$ (Table IV). Thus, the production of $C^{14}O_2$ *in vitro* is probably the result of demethylation rather than transmethylation. The codeine remaining was further identified by an appraisal of the specificity of the methyl orange technique for codeine in the manner described by Brodie. The solubility characteristics of this remaining codeine were found to be essentially the same as those of a standard codeine solution.

*Discussion.* Our *in vitro* experiments indicate that the production of $C^{14}O_2$ from codeine* labelled in the methoxy position is not a result of transmethylation. We have not definitely ruled out the possibility that the process is one of de-methoxylation. However, since Bernheim has shown that as much as 13% of codeine incubated with liver slices appeared as a phenolic-OH compound, it is probable that the process concerned is actually that of demethylation. It has been shown that codeine* is rapidly demethylated in rats *in vivo*. Approximately 20% of the injected $C^{14}$ appears as expired $CO_2$ within 2 hours. The figure representing the portion of the effective dose demethylated is presumably

TABLE III.
*In vitro* Formation of $C^{14}O_2$ from Codeine*.

| Tissue | Time of incubation, hr | Codeine* identified as $CO_2$ in mg/kg fresh tissue | % of total codeine* added |
|---|---|---|---|
| Muscle | 2 | .33 | .02 |
| ” | ” | .48 | .02 |
| Kidney | ” | 1.33 | .2 |
| ” | ” | 4.44 | .4 |
| Liver | ” | 202. | 32.0 |
| ” | ” | 350. | 43.4 |
| ” † | ” | 38.8 | 6.2 |

† This liver presented a gross aspect of chronic passive congestion.

TABLE IV.
Comparison Between $C^{14}$ and Methyl Orange Methods of Determining Codeine Content of Filtered Incubating Fluid.

| Sample | $C^{14}$ analysis µg codeine* | Methyl orange analysis µg codeine |
|---|---|---|
| Control liver | 603 | 575 |
| Incubated liver | 350 | 324 |
| ” ” | 185 | 182 |
| ” ” | 217 | 207 |

higher since within this period some 18% of the injected dose has not yet been mobilized (12). The methoxy group is oxidized to $CO_2$ and promptly excreted in the expired air. It is of interest to note that in spite of the large quantities of $C^{14}O_2$ present in the expired air, only negligible amounts are found in the tissues as $CO_2$ or $HCO_3^-$. One may assume, therefore, that in experiments dealing with the distribution of codeine* labelled in the methoxy position, the presence of $C^{14}$ due to $CO_2$-$HCO_3^-$ in the tissues does not modify the interpretation of results based on a consideration of the total $C^{14}$ content of the tissue. This does not preclude the possibility that other radioactive compounds may have been formed or that modifications of the codeine* molecule may occur which contribute to the radioactivity of the tissue.

Demethylation *in vivo* proceeds at an appreciable rate for at least 6 hours after drug administration, at which time about 40% of the injected dose has been demethylated. As long as 31 hours after such an injection, a small but measurable amount of radioactivity is still being excreted as $CO_2$.

Although the *in vitro* experiments indicate that demethylation can take place to a small extent in kidney and in muscle, these organs probably do not play a significant role in this process *in vivo*. Considering the fact that in the body the total weight of muscle may be 10 times the weight of liver, and that the capacity for demethylation of the former is only 1/500 the latter, the over-all contribution of muscle would still be only 2% that of liver. Similar calculations for kidney show that *in vivo* this organ has a capacity amounting to 0.2% that of the liver. Thus, with approximately 98% of the demethylation occurring in the liver, one may assume that the appearance of radio-$CO_2$ in the expired air is due to demethylation of codeine* in the liver. On this assumption calculations of the *in vivo* capacity on a per kg wet liver basis give figures of, respectively, 240, 174 and 220 mg codeine* demethylated in 2 hours for the animals listed in Table I. These figures are of the same order of magnitude as those found in our *in vitro* experiments indicating that the enzymatic systems involved operate efficiently *in vitro*. Although it has been demonstrated that blood serum is capable of deacetylating heroin(13), rat blood apparently has no effect on the methoxy group of codeine.

In one of our *in vitro* experiments the presence of unspecified liver damage (as reflected in a gross appearance of chronic passive congestion) markedly interfered with demethylation. It is of interest to speculate how such interference *in vivo* would modify the analgesic response to codeine. The question of whether the analgesia of codeine is due solely to its demethylated product is worthy of consideration. The analgesic effect of the intact molecule can be studied under conditions where demethylation is prevented, as in hepatectomized animals or possibly in animals with severe liver damage.

*Summary*. Radio-codeine is demethylated *in vitro* and *in vivo*, the labelled methoxy group being oxidized to $C^{14}O_2$ which is expired by the lungs. The site of the demethylation process is primarily the liver; 40% of the injected dose is demethylated within 6 hours. Transmethylation has not been demonstrated *in vitro*.

The authors wish to express their indebtedness to Professor H. H. Anderson and to Drs. H. W. Elliott and E. Leong Way for their many helpful suggestions.

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# Radio Iodine Uptake in Humans. V. Absolute Determination of Radioiodine in the Thyroid. (17697)

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In the course of an investigation on the uptake of radio iodine in humans, it became apparent that a technique was needed which would permit reliable comparison of results obtained from many patients. The development of such a technique, based on basic principles of the behavior of radiation was undertaken. The result of the development was a method of measurement which permitted not only reliable intercomparison of results, but also led directly to the determination of the absolute amount of radio iodine in the thyroid gland at the time of measurement.

From previous work(1) in which radioactivity in the thyroid was measured by placing a Geiger counter directly over the gland, it was known that many errors existed, all of which were greatly exaggerated by the proximity of the gland and counter tube. These errors could be minimized by moving the counter tube some distance from the thyroid gland, but then, due to the high background caused by the radioiodine in the rest of the body of the subject, statistical accuracy of counting suffered. By enclosing the Geiger counter in a long lead tube which shielded it from all radiation except that originating in the thyroid gland area, the background was greatly reduced and statistical accuracy restored while retaining the improved geometric arrangement of source and counter tube.

It is the purpose of this paper to present (a) the counting technique as used for dogs and human subjects; (b) the utilization of these counts for the determination of the absolute radioiodine content of the thyroid; (c) a comparison of these results with those obtained by a direct measurement of thyroid radioiodine.

*Method.* On dogs, following intravenous injections of $I^{131}$, the radioactivity in the thyroid gland was counted at distances of 25 and 50 cm, the counter being shielded from all but thyroid radiation by means of a lead tube. Total thyroidectomies were done and the glands reduced to a suitable form for preparation of beta counting samples. The dogs weighed from 16 to 22 kg and were fed a standard stock diet. Forty-eight hours after intravenous injection of 75 to 100 microcuries of $I^{131}$ in distilled water, they were anesthetized by drop ether and placed on their sides on a platform. One end of a specially constructed lead tube was placed against the thyroid gland; the correct localization of the gland was checked by palpation. The lead tube was about 60 cm long, rectangular in cross section, with internal dimensions 7.5 by 12.5 cm and wall thickness of 2.5 cm. It contained openings drilled transversely at 25 and 50 cm from the thyroid end of the tube for positioning of the counter tube. A cylindrical glass wall counter tube of active length 7.5 cm, and diameter about 2 cm was used. The tube was shielded from beta radiation by a brass sleeve 0.45 cm thick. An experimental check was made to determine the amount of scattered radiation reaching the Geiger counter from the walls of the lead tube. It was found that scatter increased the gamma ray intensity at the Geiger counter by approximately 1 to 2%, which was considered negligible for this experiment.

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Five and 10 minute counts were made at 25 and 50 cm respectively. At the completion of counting, total thyroidectomies were done by Dr. Philip Smith (John Harper Seeley Fellow, Department of Surgery). Complete removal was verified in each instance by inspection and by radioactivity check. The absolute radioiodine content of each thyroid gland was derived from the 5 and 10 minute counts described above by adjusting for the geometric system used in the experiment and utilizing previously determined absolute gamma sensitivity of the counter tube.

Adjustment for geometric correction was made by calculating the solid angle intercepted by the counter tube, treating the gland as a point source of radiation. With known radiation entering this solid angle, the total radiation from the gland was determined from the ratio of this solid angle to the solid angle of the sphere(2). Since the largest dimension of the gland was substantially smaller than the distance between the gland and counter tube, the assumption that the gland may be treated as a point source was justified. An experimental verification of this assumption was made and is described in a previous paper(1).

The absolute gamma sensitivity of the counter tube was determined as follows: a standard solution of $I^{131}$ was prepared and activity verified by absolute beta determination (see below). An aliquot of this solution was pipetted into a Stender dish and evaporated at 60°C to dryness. This was treated as a calibrated source of beta and gamma radiation. By placing the Geiger tube at known distances from this source and using the geometric correction described above, the response of this tube to gamma radiation from $I^{131}$ was determined. In this determination it was assumed that one gamma photon is emitted for each beta particle. An examination of the latest $I^{131}$ decay scheme as determined by Metzger and Deutsch(2) justified this assumption for use within the detection range of our instruments. Typical sensitivity for a Radiation Counter Laboratory 10 A tube mounted in a Nuclear Instrument Corporation probe with beta shield in place is 0.002.

The method of radioactivity determination by absolute beta assay was as follows: The glands were weighed and placed in a Waring blender with a known weight of distilled water; then the blender was run for 10 minutes intermittently to avoid excessive temperatures. The mixture was removed and the blender cleared with a measured weight of distilled water. The mixture was checked by weighing to determine water loss by evaporation. Aliquots were weighed out in Stender dishes, and usually 3 samples of a given weight range and 4 different weight ranges were used. These were dried under partial vacuum in an oven at 70°. Radioactivity determinations by beta counting were made, using a thin mica window counter in a controlled geometric system. A self-absorption curve for these dried specimens of macerated thyroid tissue was obtained by plotting count per unit time against weight. By correcting for self-absorption of the specimen, window absorption, and geometric system, as well as other standard counting corrections(3), the absolute activity per g and total activity in the gland were determined. This absolute beta technic, properly adjusted, was used by us in the Iodine Intercomparison Surveys (No. 2 and No. 3) sponsored by the National Bureau of Standards. Since our determinations in these surveys agreed with the nationally accepted values within 5 and 10% respectively, we believe that this technic gives a reasonably accurate determination of the radioiodine content of the thyroid.

To date the opportunity to make similar observations in a human subject has presented itself only once. The patient was a female, 24 years old, with a moderately enlarged adenomatous gland. A dose of 505 microcuries of $I^{131}$ was given by mouth 69 hours before counting. Counts were obtained with a lead tube designed for use with human sub-

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2. Metzger, F., and Deutsch, M., *Physical Rev.*, 1948, v74, 1640.

3. Wilson, D., *et al.*, *Preparation and Measurement of Isotope Tracers: A Symposium Prepared for the Isotope Research Group*, J. W. Edwards, Ann Arbor, Mich., 1946.

TABLE I.

Values for $I^{131}$ Content of Thyroid Gland as Determined by Direct Beta Assay and by *In Vivo* Distance Counting with Lead Tube.

| | | $I^{131}$ content of thyroid | | | | | Direct assay minus *in vivo* counting | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | By *in vivo* counting | | | | | | | |
| | | | At 25 cm | | At 50 cm | | At 25 cm | | At 50 cm | |
| Subject | $I^{131}$ dosage, μc | By direct beta assay, μc | Mean, μc | Coeff. of variation,* % | Mean, μc | Coeff. of variation,* % | Absolute diff., μc | Diff. direct assay, % | Absolute diff., μc | Diff. direct assay, % |
| Dog 1 | 75 IV | 4.08 | 3.88 | 12.0 | † | † | + .20 | + 4.9 | † | † |
| 2 | 75 | 3.74 | 3.21 | 6.7 | † | † | + .53 | +14.2 | † | † |
| 3 | 100 | 7.83 | 7.41 | 5.9 | 8.44 | 20.0 | + .42 | + 5.4 | — .61 | — 7.8 |
| 4 | 100 | 14.30 | 13.70 | 1.9 | 12.33 | 5.2 | + .60 | + 4.2 | +1.97 | +16.0 |
| 5 | 100 | 7.09 | 5.59 | 3.8 | 6.15 | 11.3 | +1.50 | +21.2 | + .94 | +13.3 |
| Patient 1 | 505 *per os* | 59.67 | 58.31 | 2.0 | 65.9 | 4.3 | +1.36 | + 2.3 | —6.23 | —10.4 |

* Coefficient of variation equals standard deviation divided by mean, expressed as percent.

† These counts were so close to background counts that they were statistically unreliable.

jects and described previously in this series of studies(1). Total thyroidectomy was performed 1 hour after counting. The radioiodine content of the gland was determined by the method of absolute beta assay described above.

*Results.* A summary of the pertinent information obtained in the experiments on 5 dogs and 1 patient is presented in Table I. All the radioactivity figures listed in this table are corrected for radioactive decay as of the time of administration. The quantity of $I^{131}$ administered is indicated in the second column. At the left, under the heading "$I^{131}$ Content of Thyroid," are seen the results in microcuries of absolute beta assay carried out after operative removal of the glands. At the right under this heading are seen the results of long distance measurement of gamma sensitivity. The $I^{131}$ content of each gland as determined from measurements made at 25 cm from the thyroid is listed under the left side of this heading. Similar determinations made at 50 cm appear at the right. At the right of each of these columns is given the ratio of the standard deviation to the net total count, a measure of statistical validity of the counting known as the coefficient of variation. The statistical validity of the determinations at 50 cm for dogs No. 1 and 2 was so poor that they were discarded.

Under "Direct Assay Minus *In Vivo* Counting," the $I^{131}$ content of the removed gland as determined by absolute beta assay was taken as the standard. The reliability of the results of long distance counting was determined by comparison with this standard. The differences between the standard values and the determinations from long distance counting are listed here both in microcuries and in percent. In 4 cases the agreement of the 25 cm determination with the standard was of the order of 5%. In the fifth case, agreement was within 14.2%, and in the sixth case, agreement only within 21.2% was obtained.

In general, the agreement of the determinations made at 50 cm with the standard was not as good as the agreement obtained between the 25 cm determinations and the standard, the range being 7.8 to 16.0%. As has been indicated above, the statistical validity of the 50 cm determinations was not as reliable as that obtained for the 25 cm counts.

*Discussion.* The method of determining the $I^{131}$ content of the thyroid gland by long distance gamma counting as herein described is based on sound physical principles. To determine whether the application of these principles in this method is valid, an independent method of assay of thyroid $I^{131}$ was used, namely, absolute beta counting. From the results, we believe that the validity of the analysis used in setting up our long distance counting technic is demonstrated. In the experimental data for the 25 cm counts, the maximal deviation from the standard was of the order of 20%, with the majority about 5%. At 50 cm, with its lower statistical reliability, the deviations ranged from 8 to 10%. For biological purposes, this method in its present stage of development can be considered quantitative and applicable whenever determinations of $I^{131}$ content of the thyroid gland *in situ* are desired.

Statistical accuracy of *in vivo* measurements, such as employed in this technic, can be improved upon by (a) an increase in counting time, (b) a decrease in background radiation, (c) an increase in dose of radioactive material, and (d) an increase in amount of radiation utilized in counting. In the experiments for each determination, a 20 minute counting period broken into 5 or 10 minute runs was used. It was found that a 500 second count was about the maximal time the patient could tolerate the relative immobility required. The reduction in background achieved by the lead shield tube appears maximal for this avenue of improvement. Because of potential radiation damage, increase in dose should be avoided. The most rewarding approach lies in increasing the utilization of available radiation. The absolute gamma sensitivity of the counters used in this work was about 0.002, which is typical of commercially available counter tubes of the Geiger type. However, the crystal type counter now in the process of development has an inherent gamma sensitivity of 0.25 or better, which would give a hundredfold increase in counts. When these high efficiency counters become available, the accuracy of the long distance counting technic can be greatly improved.

*Summary.* A method of counting at a distance over the thyroid was used in animal and human subjects. This method involves the shielding of the Geiger counter tube from all radiation except the thyroid gland, by enclosing the counter in a long lead tube opening over the thyroid. The statistical error inherent in long distance counting is thereby effectively reduced. Independent beta assays were done on the thyroids of 5 dogs and 1 human adult who were subjected to total thyroidectomy immediately after counting with the above technic. Comparison of radioactivity of these glands, as determined by beta assays and by long distance gamma counting, showed a maximal deviation of the order of 20%, with the majority agreeing within about 5%.

Suggestions are made regarding improvement of *in vivo* measurements such as employed in this technic.

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# Action of Proteolytic Enzymes on Collagen. (17698)

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In studies on comparative properties of collagens from various sources it was observed that samples prepared by mild processes were generally resistant to the action of common proteolytic enzymes with the exception of pepsin. Collagens prepared by more drastic means and commercial hide powder were highly susceptible to attack by the proteolytic enzymes. It was apparent that the use of gelatin, hide powder, or any modified collagen as a substrate for the detection of so-called "collagenase" activity would not be valid. In our laboratory *Clostridium histolyticum* filtrates and pepsin did actively solubilize collagen. *Clostridium perfringens* (*BP6K*) filtrates reputed by Maschmann(1), Macfarlane *et al.*(2), and Oakley *et al.*(3) to contain a "collagenase" were 10–20-fold less effective on collagens than equal quantities of *Cl. histolyticum* filtrates although hide powder and gelatin were attacked at a rapid rate. Jennison(4) reported that *Cl. perfringens* did not attack collagen from cattle tendon and cattle hide although his data are of a qualitative nature. It has been reported(5,6) that trypsin pretreatment results in greatly increased solubilization of collagens at the shrinkage temperatures (50-70°C). In the present work collagens from several sources revealed this effect after pretreatment with trypsin and papain. However, data are presented indicating that residual traces of enzyme are responsible for the increased solubilization. Heating native collagen in aqueous solution at 60-70°C renders it susceptible to tryptic action. In the present study it is shown that heat or urea denaturation of cattle Achilles tendon collagen results in increased digestion by trypsin, papain, and *Cl. perfringens* enzyme preparations. The increase in amount digested is approximately the same for all 3 enzymes.

*Experimental procedure.* Collagen† was prepared from Achilles and tail tendons of various animals by simple mechanical separation and shredding in the cold in a Waring Blendor with subsequent washing and extraction with cold acetone, alcohol, and ether. More highly purified material was attacked by proteases to the same extent. *Cl. histolyticum* and *Cl. perfringens* enzymes were produced in culture filtrates on a medium previously described(8). Incubation of collagen

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† We are grateful for materials which were generously supplied to us as follows:

Kangaroo tail tendon by Johnson and Johnson Co., Brunswick, N.J., and by Dr. T. Salo, M. I. T. These were air-dried sinews.

Steer hide collagen by Dr. R. M. Lollar and Dr. Peter R. Buechler, Tanners' Research Council Laboratory, University of Cincinnati. Two samples were obtained: one prepared according to a published procedure(7) by treatment with $Ca(OH)_2$ and trypsin, and the second by brief extraction at 0°C with 10% NaCl and then with acetone.

Sodium "Lorol" sulfate PT, a wetting agent, was supplied by E. I. duPont de Nemours and Company, Wilmington, Del.

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TABLE I.

Solubilization of Collagens by Enzymes.*

| Incubation time, hr | Enzyme<br>Trypsin, % | Cryst. trypsin, % | Papain, % | Cryst. chymotrypsin, % | *Cl. perfringens* pptd. enzyme, % | *Cl. perfringens* filtrates, % | *Cl. histolyticum* filtrates, % | Pepsin, % | |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 59.4 | 60.3 | 53.8 | 57.7 | 20.6 | 22.5 | 50.4 | 7.7 | Hide powder |
| 24 | | | | | | | | 88.5 | |
| 1 | 5.4 | 2.3 | 3.3 | 1.4 | 0.0 | 0.0 | 2.0 | 0.0 | Cattle Achilles tendon |
| 24 | 8.2 | 1.8 | 3.0 | 4.3 | 12.4 | 18.9 | 51.4 | 94.7 | |
| 72 | 4.9 | 6.8 | 6.6 | 5.6 | 22.1 | 47.2 | 94.7 | | |
| 1 | 3.8 | | | | | 1.8 | | | Pig Achilles tendon |
| 24 | 5.7 | | | | | 21.4 | 97.4 | 95.7 | |
| 72 | 5.0 | | | | | 63.1 | | | |
| 1 | | 2.4 | 1.2 | 0.0 | 2.9† | 0.0 | 1.3 | | Kangaroo tail tendon |
| 24 | 4.9 | 1.2 | 2.2 | 3.3 | 6.9† | 15.2 | 23.8 | 80.8 | |
| 72 | 7.9 | 4.4 | 5.4 | 4.4 | 18.4† | 29.5 | 49.8 | | |
| 1 | | | | | | 1.1 | | | Rat tail tendon |
| 24 | 4.9 | | | | 36.9† | 43.6 | 91.8 | | |
| 72 | | | | | 68.3† | 87.3 | | | |

* Enzymes used in the following quantities:

| | |
|---|---|
| Trypsin (Merck 1:250) | 1.0 mg |
| Crystalline trypsin | 0.1 |
| Papain (KCN activated) | 1.0 |
| Crystalline chymotrypsin | 0.2 |
| *Cl. perfringens* pptd. enzyme | 2.0 |
| *Cl. perfringens* filtrates | 3.0 ml |
| *Cl. histolyticum* filtrates | 0.2 |
| Pepsin (U.S.P.) | 1.0 mg |

† 5.0 mg.

TABLE II.
Solubilization of Collagen After Enzyme Pretreatment.

| Treatment of Collagen after incubation with trypsin or papain | | | Collagen residue dissolved by heating, % |
|---|---|---|---|
| Washing agent | Heated at °C | Heating medium | |
| | Trypsin.* | | |
| Distilled water | 66 | Distilled water | 94 |
| M/10 $Na_2HPO_4$ | ” | ” ” | 14 |
| ” $NaH_2PO_4$ | ” | ” ” | 16 |
| ” NaOH | ” | ” ” | 8 |
| 2% Na ‘‘Lorol’’ sulfate | ” | ” ” | 5 |
| Distilled water | ” | 0.1% $H_2O_2$ | 94 |
| ” ” | 70 | Distilled water | 94 |
| ” ” | 75 | ” ” | 62 |
| ” ” | 80 | ” ” | 28 |
| ” ” | 85 | ” ” | 12 |
| ” ” | 100 | ” ” | 12 |
| | Papain.† | | |
| ” ” | 66 | ” ” | 50-90 |
| M/10 $Na_2HPO_4$ | ” | ” ” | 49 |
| ” NaOH | ” | ” ” | 5 |
| Distilled water | 75 | 0.01% $H_2O_2$ | 5 |
| ” ” | 70 | Distilled water | 81 |
| ” ” | 75 | ” ” | 94 |
| ” ” | 80 | ” ” | 94 |
| ” ” | 85 | ” ” | 94 |
| ” ” | 95 | ” ” | 94 |
| ” ” | 100 | ” ” | 27 |

* 1 mg per tube.
† (activated with KCN) at 2 mg enzyme per tube.

with enzymes other than pepsin was conducted at 37° C in tubes containing 100 mg of substrate and 5 ml of 0.05 M phosphate buffer at pH 7.2. Papain was activated with 0.01 M KCN. Pepsin digestion was conducted in 10 ml of HCl solution at pH 2. A reasonably accurate measure of digestive action of enzymes was obtained by weighing the washed and dried residue of the collagen. Enhanced ease of solubilization of collagen at 66-75°C after enzyme pretreatment was determined after washing the collagen residue several times with distilled water or other solution. The residues were resuspended in water or other solution and placed in a water bath at designated temperature 20 minutes. The degree of solubilization was measured as before. Heat denaturation of a percentage of the collagen was accomplished by heating 100 mg in 5.0 ml of water at 65°C for 20 minutes. Urea denaturation of a percentage of the collagen was accomplished by suspension of 100 mg in 5.0 ml of 4 M, 5 M, or 6 M urea at 37°C for 24 hours. After denaturation the supernatant was removed and residues washed. The residues were then incubated with the enzymes.

*Results.* It is obvious as shown in Table I that hide powder is highly susceptible to attack by all the proteases tested. Extensive and rapid digestion of all collagen samples was obtained only with *Cl. perfringens* and *Cl. histolyticum* filtrates, and pepsin. Collagen preparations from chicken tendon, sheep tendon, turtle subcutaneous membrane, cattle tail tendon, rat tail tendon, kangaroo tail tendon, cattle *chordae tendinae,* cattle hide, and cattle bone showed resistance to trypsin and susceptibility to *Cl. histolyticum* enzyme. Other experiments not noted in the table demonstrated that collagen prepared by relatively drastic procedures and thus possibly modified was much more susceptible to digestion by all the enzymes, especially during the initial stages of the digestion.

Pretreatment with trypsin, crystalline trypsin, and papain increased the ease of solubili-

TABLE III.
Digestion of Cattle Achilles Tendon Collagen After Heat Treatment and Incubation with Urea.

| Enzyme | Pretreatment of Collagen | Solubilization, % |
|---|---|---|
| Trypsin* | 0 | 2.5 |
| | 20 min. at 65°C | 68.2 |
| Papain† | 0 | 0 |
| | 20 ” ” ” | 68.2 |
| *Cl. perfringens* pptd. enzyme‡ | 0 | 6.2 |
| | 20 ” ” 76 | 71.8 |
| Trypsin* | Distilled water 24 hr at 37°C | 3.3 |
| | 4 M urea ” ” ” ” | 0 |
| | 5 ” ” ” ” ” | 35.9 |
| | 6 ” ” ” ” ” | 59.0 |
| Papain† | Distilled water ” ” ” ” | 1.4 |
| | 4 M urea ” ” ” ” | 6.6 |
| | 5 ” ” ” ” ” | 31.6 |
| | 6 ” ” ” ” ” | 58.8 |
| *Cl. perfringens* pptd. enzyme‡ | Distilled water ” ” ” ” | 16.4 |
| | 4 M urea ” ” ” ” | 10.5 |
| | 5 ” ” ” ” ” | 43.4 |
| | 6 ” ” ” ” ” | 70.0 |

* Trypsin at 1 mg per tube.
† Papain (KCN activated) at 2 mg per tube.
‡ *Cl. perfringens* pptd. enzyme at 2 mg per tube.
Incubation with enzyme 24 hr at 37° C ± 0.5°.

zation of collagen at 66 to 75°C after thorough water washing. Crystalline chymotrypsin and *Cl. perfringens* enzymes did not show appreciable effect. As shown in Table II washing with M/10 solutions of phosphate or sodium hydroxide practically eliminated the solubilization effect. Na "Lorol" sulfate which inhibits trypsin destroyed the solubilization effect. At the same time peroxide did inhibit the solubilization due to papain but not that due to trypsin. Applying a post-enzymic heating treatment of 66-100°C to the collagen residues demonstrated an inhibition with rise of temperature. Collagen from different sources displayed these characteristic reactions. It appears that the reported ease of solubilization of collagen after trypsin pretreatment is an effect of quantities of enzyme which are difficultly removed from collagen by washing in water at room temperature.

Table III shows that heating collagen in water at 65°C or treatment with urea increased susceptibility to 3 different proteases to the same extent. Collagen from chicken tendon, pig tendon, sheep tendon, turtle subcutaneous membrane, cattle tail tendon, cattle *Chordae tendinae,* cattle hide, and cattle bone after similar heat treatment became susceptible to tryptic attack.

*Summary.* 1) The use of hide powder as a substrate in the demonstration of collagenase activity is not valid.

2) Collagens from several sources, prepared by methods designed not to alter properties, are resistant to the action of trypsin, chymotrypsin, and papain. The collagens are readily attacked (solubilized) by the proteolytic enzyme(s) of *Cl. histolyticum* and by pepsin. The proteolytic enzyme(s) of *Cl. perfringens* filtrates are 10–20-fold weaker than those of *Cl. histolyticum* filtrates in the degradation of collagen.

3) The reported increased solubilization of collagens in water at the shrinkage temperature (68–70°C) after incubation with enzymes can be attributed to residual enzyme.

4) Denaturation of collagen by heat and urea produces a general susceptibility to common proteolytic enzymes.

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# Histochemical Determination of Ribose Nucleic Acid in Vertebrate Tissues Following Extraction with Perchloric Acid.* (17699)

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The standard procedure for determining histochemically the presence of ribonucleic acid in cells consists of treating sections with a buffered solution of ribonuclease prior to staining. The basophilic staining property of the ribonucleic acid is lost following digestion with the enzyme. The preparation of ribonuclease is beyond the scope of most biological laboratories and the cost of the prepared enzyme is prohibitive for routine investigation. Since the determination of the ribonucleic acid content of cells has become an important adjunct in histochemical and pathological studies, a more convenient substitute for ribonuclease is desirable. Using photocolorimetric estimation, Ogur and Rosen(1) obtained data which indicate that prolonged contact in the cold with perchloric acid extracts pentose nucleic acid from plant tissue residues. Seshachar and Flick(2), studying *Chilodonella,* report that prolonged treatment with cold perchloric acid extracts PNA from this protozoan. This investigation deals with the adaptation of a method for substituting cold perchloric acid for ribonuclease in the determination of ribonucleic acid in vertebrate tissues.

*Methods.* Treatment of deparaffinized sections on slides with cold perchloric acid showed no significant effects when compared with control sections. The following methods were then used:

A. Tissues from rats and cats (liver, autonomic ganglia, sensory ganglia, pancreas, thyroid and parotid glands) were fixed in Zenker's Fluid for 8-15 hours and washed in running water for 2 hours. One-half of each block of tissue was placed in 10% perchloric acid from 5 to 24 hours at 5°C. Following preliminary study, the 18 hour time interval was used exclusively. The other half of each block of tissue was placed in water for a similar length of time at the same temperature to serve as controls. The tissues were again washed in running water for 2 hours, dehydrated, imbedded and sectioned in the usual manner. Sections from both perchloric acid treated tissues and from controls were placed on each slide and stained with Mallory's phloxine-methylene blue.

B. Tissues similar to those in section A were fixed in Zenker's Fluid for 8-15 hours, washed in running water for 2 hours and sectioned at 15 microns with the freezing microtome. Half the sections from each block of tissue were placed in 10% perchloric acid for 18 hours at 5°C, the other half in water at 5°C for the same length of time. The sections were rinsed in water, stained with 1% toluidine blue for 5 minutes, counterstained with 0.5% alcoholic eosin for 15 seconds, differentiated in 95% alcohol and mounted in glycerin.

*Autonomic and Sensory Ganglia.* In the control sections of autonomic and sensory ganglia, the chromidial bodies and nucleoli are stained with the basophilic stains (Fig. 1 & 3). The changes induced by treatment with cold perchloric acid were very striking. The chromidial bodies and the nucleoli were completely unstained by the toluidine blue or methylene blue, whereas the chromatin material of the neurological cells was unaffected. (Fig. 2 & 4). The nucleolus and the nucleolar membrane in the treated sections appeared acidophilic. Similar results were obtained in ventral root cells by Gersh and Bodian(3) after treating sections of the spinal cord of rhesus monkeys with a buffered solution of ribonuclease.

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* This investigation was supported by a research grant from the National Heart Institute, U. S. Public Health Service.

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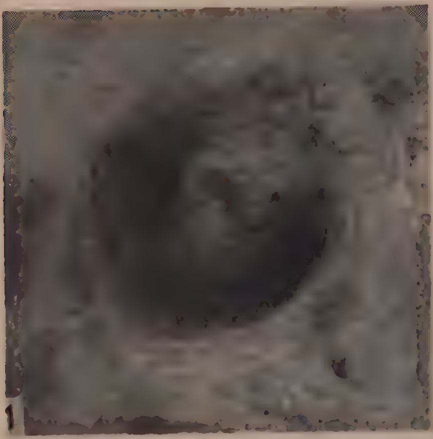
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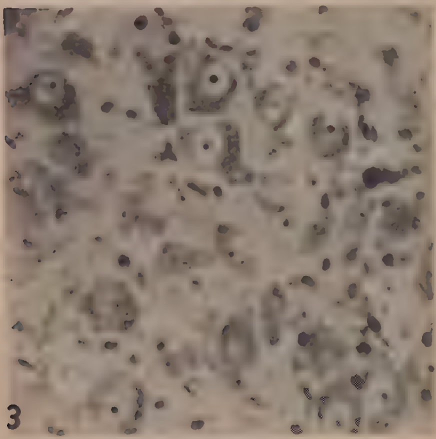
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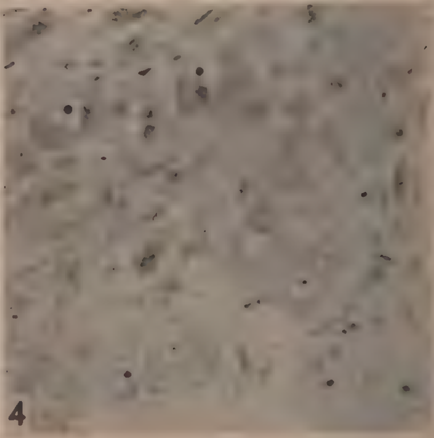
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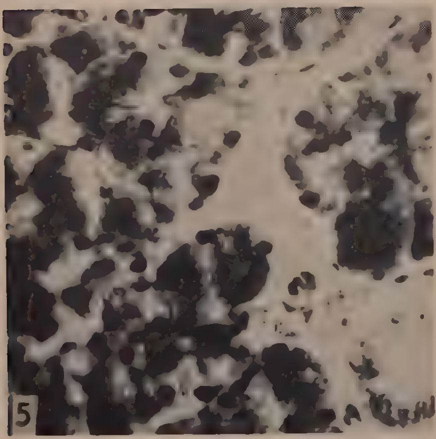
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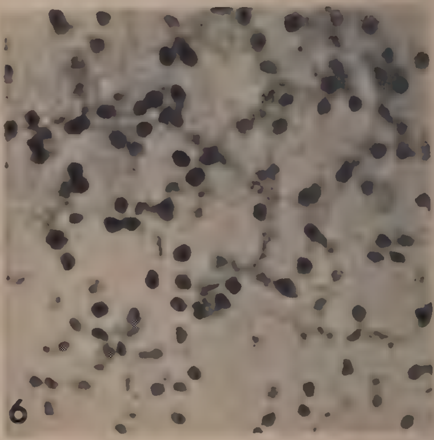
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FIG. 1. Frozen section of superior cervical ganglion stained with toluidine blue and eosin. Ocular 10×, obj. 2 mm.

FIG. 2. Frozen section of superior cervical ganglion treated with cold 10% perchloric acid and stained with toluidine blue and eosin. Ocular 10×, obj. 2 mm.

FIG. 3. Paraffin section of superior cervical ganglion stained with Mallory's phloxine-methylene blue stain. Ocular 10×, obj. 8 mm.

FIG. 4. Paraffin section from same slide as above of superior cervical ganglion which had been pre-treated with cold 10% perchloric acid and stained with Mallory's phloxine-methylene blue stain. Ocular 10×, obj. 8 mm.

FIG. 5. Paraffin section of parotid gland stained with Mallory's phloxine-methylene blue stain. Ocular 10×, obj. 8 mm.

FIG. 6. Paraffin section from same slide as above of parotid gland which had been pre-treated with cold 10% perchloric acid and stained with Mallory's phloxine-methylene blue stain. Ocular 10×, obj. 8 mm.

*Pancreas.* The cells of the pancreatic acini have a characteristic two-toned appearance when stained with basophilic and acidophilic stains. The basal zones of the cells, in which the nuclei are located, appear striated or homogenous and stain basophilically. The apical zones are acidophilic. Following treatment with cold perchloric acid the basal portions of the cells lose their basophilia, except for the chromatin material of the nucleus. Similar results are obtained by treating sections with ribonuclease.

*Thyroid Gland.* Dempsey and Singer(4) reported that the basophilia of the follicular thyroid epithelium with the eosin-methylene blue stain is an indicator of the presence of ribose-nucleoprotein. In their preparations, the basophilia of the follicular epithelium was lost following treatment of the sections with ribonuclease. Prolonged treatment with cold perchloric acid produces identical results. The cytoplasm of the follicular cells becomes slightly acidophilic although the chromatin material retains its basophilic staining properties.

*Liver.* The intensity and the frequency of the basophilic staining of the cytoplasm in hepatic cells is extremely variable, due to dietary, metabolic and other factors. In the normal healthy animal, some hepatic cells always show a basophilic staining reaction which disappears following treatment of the sections with ribonuclease. Similar loss of basophilia has also been induced in the liver of the rat and the cat following the described treatment with perchloric acid. Here, also, the nuclear basophilia remains unchanged.

*The Parotid Gland.* The cytoplasm of the albuminous secreting cells of the parotid gland stains lightly basophilic, apparently due to ribose-nucleoprotein. The basophilia is usually most intense in the basal portions of the cells (Fig. 5). Following extraction with cold perchloric acid, the basophilic staining reaction of these cells is completely lost (Fig. 6).

*Conclusions.* The data obtained in these experiments indicate that perchloric acid may be substituted for ribonuclease in the histochemical determination of ribonucleic acid. The results obtained by the use of perchloric acid were identical to those obtained by the use of ribonuclease as the digestive agent. The most consistent results were obtained with frozen sections, apparently because of better diffusibility.

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# The Effect of Cortisone on the Spleen in Mice.[*] (17700)

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During the course of an investigation on the effect of Cortisone on the reticulo-endothelial system in mice, it was noted that doses of Cortisone of the comparable order of magnitude employed therapeutically significantly influenced the size of the spleen. Forty 20-25 g Swiss-albino male mice from a Bartonella-free herd were controlled for enteric and upper respiratory infection and divided into two equivalent groups according to weight. Diet and housing were controlled, each mouse was housed individually and food and water intake grossly noted. The Cortisone-treated mice received daily intraperitoneal injections of 2 mg total divided into 2 equal doses of one mg each, administered 8 hours apart.

The injection schedule was followed for 6 days with daily sacrifices given below. Animals were sacrificed 12 hours after the preceding injections. The control group of mice received the suspending diluent without Cortisone administered in the same volume and manner as the treated group and sacrificed similarly. At sacrifice each animal was subjected to a complete autopsy, gross and microscopic examination made of all organs and tissues. For the purpose of the present report the spleens were weighed and sections prepared. The doses of Cortisone, schedule of sacrifice and spleen findings are all given in Table I.

*Microscopic findings.* Sections of spleens from Cortisone-treated animals revealed smaller Malphigian bodies and fewer mononuclear and lymphocytic cells in the pulp. No abnormal cells nor evidence of any degeneration was noted. The spleens of the Cortisone-treated animals appeared similar to those of the controls in all respects with the exception of the changes noted above.

*Summary.* 1. Cortisone causes a significant reduction in the spleen size of mice. The average spleen size of the Cortisone-treated mice was 21% of the controls.

2. As little as 4 mg of Cortisone during a 2 day period produced this effect. Continued Cortisone treatment did not increase the original depletion, indicating that the effect is rapid and maximal.

3. Histologically there was a reduction in the size of the Malphigian bodies and in the number of cellular elements in the pulp in the Cortisone-treated group.

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TABLE I.

| A. Cortisone Treated<br>Dose: 1 mg 2 times daily | | | | B. Controls. No Cortisone<br>Diluent: 2 times daily | | |
|---|---|---|---|---|---|---|
| 4 mice in each experiment. | | | | | | |
| No. of days treated | Total dose per mouse, mg | Inj. per mouse | Avg wt per spleen, mg | No. of days treated | Inj. per mouse | Avg wt per spleen, mg |
| 2 | 4 | 4 | 37 | 2 | 4 | 204 |
| 3 | 6 | 6 | 44 | 3 | 6 | 156 |
| 4 | 8 | 8 | 47 | 4 | 8 | 198 |
| 5 | 10 | 10 | 39 | 5 | 10 | 196 |
| 6 | 12 | 12 | 33 | 6 | 12 | 196 |
| Avg wt per spleen 40 mg<br>Range: 15 to 55 mg | | | | Avg wt per spleen 190 mg<br>Range: 53 to 208 mg | | |

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[*] The Cortisone for this study was allocated by The Committee on Cortisone of the National Research Council.

# Effects of SC 1950 (2,6 Dimethyl Diethyl Piperidinium Bromide) on Peripheral Circulation.* (17701)

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Stimulation of the peripheral circulation is of decided benefit in the treatment of a number of clinical conditions, especially Raynaud's disease, reflex sympathetic dystrophy, and vasospasm accompanying arteriosclerosis obliterans as well as thromboangiitis obliterans. Such an increase in the blood flow to the extremities can be accomplished through medication with vasodilating drugs, however always at the risk of certain undesirable side reactions, as for instance, sleepiness, or dizziness. It was therefore decided to determine whether or not the new quaternary amine 2,6 dimethyl diethyl piperidinium bromide (SC 1950),† synthesized by I. C. Winter and his associates, increases the blood flow to the extremities and thus exerts a significant influence upon peripheral circulation. This drug, described as a ganglionic blocking agent, has proved in animal experiments to be several times as potent as tetraethylammonium bromide(1) and results in man and dog in lowering of blood pressure, reduction of tonus, and concomitant increase in the skin temperature of the toe(2).

*Methods and materials.* The effectiveness of SC 1950 was studied in 5 normal individuals and 21 patients with peripheral arterial disease. Of the normal individuals, 3 were males, 2 females, the average age in this group amounting to 28 years (21 to 42). Of the 21 patients with arterial disease, 14 were males, 7 females, the average age in this group amounting to 33 years (24 to 55). In 10 cases the diagnosis was arteriosclerosis obliterans, in 5 essential benign hypertension, in 4 thromboangiitis obliterans, while hypertension secondary to pyelonephritis, and to periarteritis nodosa was noted in one case each. The experiments were conducted under standard reproducible conditions. All subjects were dressed in hospital gowns and examined after 45 minutes of rest. Observations were made in an air conditioned room with a temperature of 25°C ± 1.5°, the air velocity amounting ordinarily to less than 10 feet per minute.

Changes in the volume of the extremities were determined by means of a digital pneumoplethysmograph(3) and a digital cup was fastened to the tip of the right or left second toe or finger. Plethysmograms were standardized so that volume changes were recorded in cu mm/5 cc per sec. Blood flow to the extremities was ascertained through venous occlusion(4) and a collecting cuff placed at ankle or wrist and in selected cases at finger or toe. In order to establish the skin temperature, thermocouple junctions were fixed to the skin of the extremities with cellulose tape, and readings from a 12-point electronic potentiometer were recorded every 15 seconds. These observations were exact within ± 0.25°C, and the accuracy of the instrument employed was frequently tested with a National Bureau of Standards mercury thermometer, exact to within 0.1°C. Blood pressure was measured by the standard auscultatory or the plethysmographic method, or in

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The valuable technical assistance of Grayce S. Fleming is sincerely acknowledged.

† Drug gratuitously provided by the Research Laboratories of G. D. Searle and Co.

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selected cases additional continuous tracings were taken using the Sphygmotonograph(5). Oscillometric readings were recorded by means of the Collen's Sphygmo-oscillometer. The cardiac rate was calculated from the plethysmogram. As a rule, SC 1950 was administered through an intravenous tube by slow injection over a period of 15 minutes and in a dose of one-half mg per kg of body weight. Control determinations were made with normal saline introduced by intravenous drip at the rate of 10 drops per minute.

*Results: Effect of SC 1950 on Blood Pressure, Pulse Rate and Respiratory Rate.* Blood pressure was consistently lowered in normal individuals as well as in patients with early or advanced peripheral arterial disease. Among normal individuals the blood pressure dropped from an average of 132/80 (118/76 to 140/90) before injection to 85/63 (60/40 to 100/68) 25 minutes after instillation of SC 1950. Consequently the pulse pressure fell from an average of 52 mm Hg before to 22 mm Hg, 25 minutes after injection. In 2 normal individuals 40 mg were administered in 3 minutes and the blood pressure reached its lowest level within 8 minutes; injection extending over a period of 15 minutes, on the other hand, resulted in a less pronounced fall of pressure. The hypotensive state lasted for a variable period of time, the average for the entire group of 26 subjects amounting to 55 minutes. The three normal individuals, however, showed lowered blood pressure readings for more than 150 minutes. In patients with hypertension and obliterative arterial disease the blood pressure was also lowered by the drug. However, the response of patients of this type to comparable doses showed considerable variations and necessitated great care in the administration of SC 1950. For instance, in the case of a patient with essential benign hypertension the blood pressure fell from 180/100 before injection to 70/60 following administration of 40 mg of the drug. The patient became ashen gray and complained of precordial pressure, but 10 minutes after arms and legs had been elevated the blood pressure returned to 100/70. As the patient's renal function, determined by clearance and phenolsulfonphthalein tests proved to be normal, his unusual sensitivity is not readily explained.

In normal individuals (Fig. 1) as well as in patients with peripheral arterial disease the Flack test gave ordinarily positive values as long as the blood pressure was reduced, similarly as in Hecht and Anderson's study on dibenamine(6); however, the Flack test turned negative as soon as the blood pressure once again reached a normal level. Postural hypotension appeared generally a few minutes after completion of injection, but became less marked when the blood pressure began to return to normal. The pulse rate ordinarily was accelerated following intravenous injection of SC 1950. In 3 of the 5 normal individuals the pulse rate increased from an average of 102 (80 to 134) before injection, to 132 (92 to 148) within 10 minutes after injection. With the return of the blood pressure to normal, the pulse rate reverted to its pre-injection level. In patients with arteriosclerosis obliterans or with essential hypertension the pulse rate showed generally little change following injection of the drug. This obvious dissimilarity in the response to SC 1950 may well have to be explained on the basis of varying degrees of blocking of the autonomic nervous system obtained in different individuals.

The respiratory rate was studied in all 26 subjects, and 9 times no significant change was noted; in the remaining 17 cases respirations became shallow following administration of SC 1950, most noticeably so in conjunction with a marked decline in blood pressure.

*Effect of SC 1950 on Pulsations of Extremities and Digits.* In the 5 normal individuals the pulse volume of the digit showed a marked increase under the influence of SC 1950. A fair rise in pulse volume is ordinarily encountered in patients with moderate peripheral arterial disease, and in one typical case in that group (Fig. 2) the pulse volume rose within 15 minutes from 0.2 cu mm/5 cc of tissue to 4.0 cu mm. Among patients with advanced

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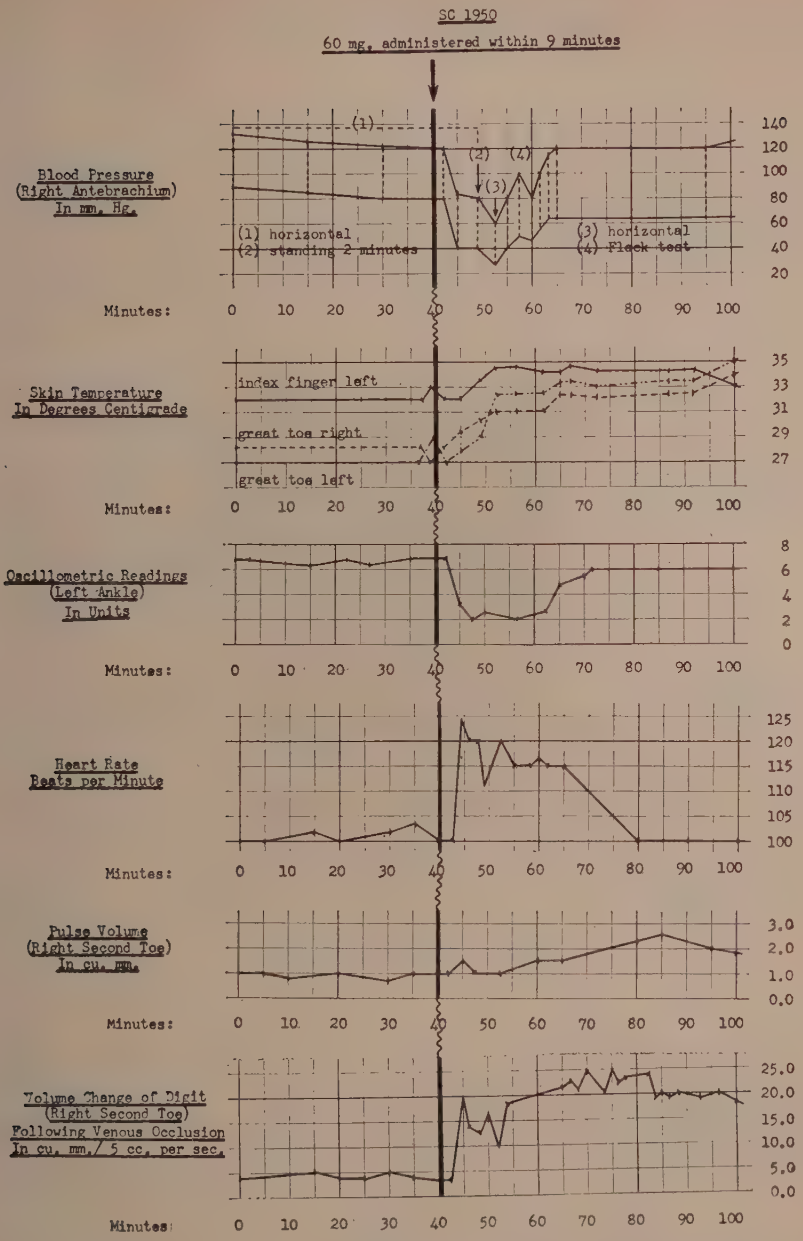

FIG. 1.

Effect of SC 1950 on peripheral circulation in normal male, age 32. Definite fall in antebrachial blood pressure to a hypotensive level, with postural hypotension as well as positive Flack test 17 min. after intravenous inj. of 60 mg SC 1950. The skin temp. of dorsum of left toe increased in 60 min. from 27.1 to 34.0°C. Oscillometric readings showed a decided fall. The heart rate rose sharply during the hypotensive phase. Amplitude of pulsations in the toe was accentuated as blood pressure returned to normal. Volume change following venous occlusion (relative toe flow) increased about 8 times.

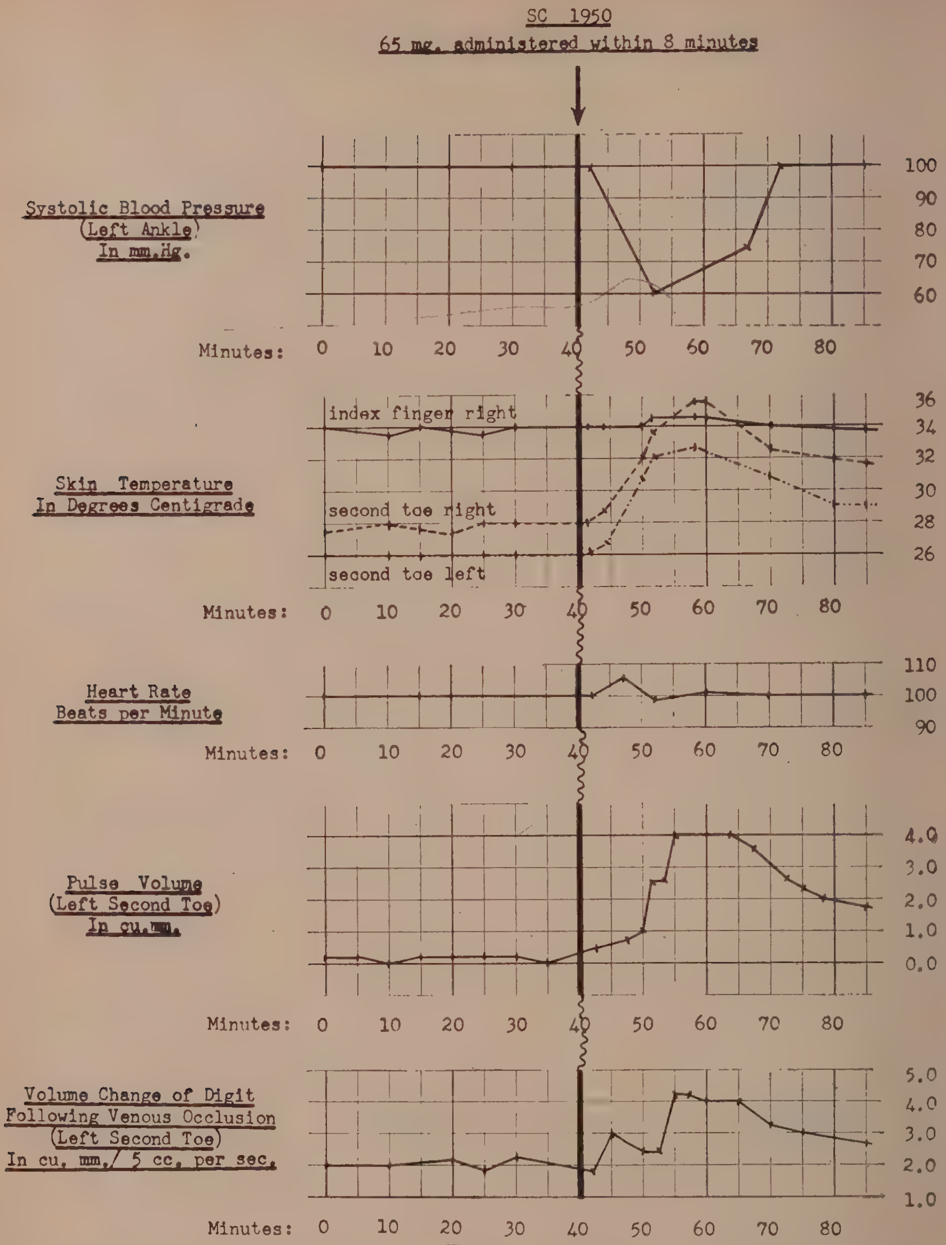

FIG. 2.

Effect of 65 mg SC 1950 intravenously, on peripheral circulation of a 34 year old male with early thromboangiitis obliterans of left leg. Pain in left calf on walking 4 blocks. Following inj., systolic blood pressure at left ankle fell 40 mm Hg. Skin temp. in left toe increased less than in the right. Heart rate slightly accelerated. Pulse vol. rose noticeably. Volume change following venous occlusion (relative toe flow) moderately increased.

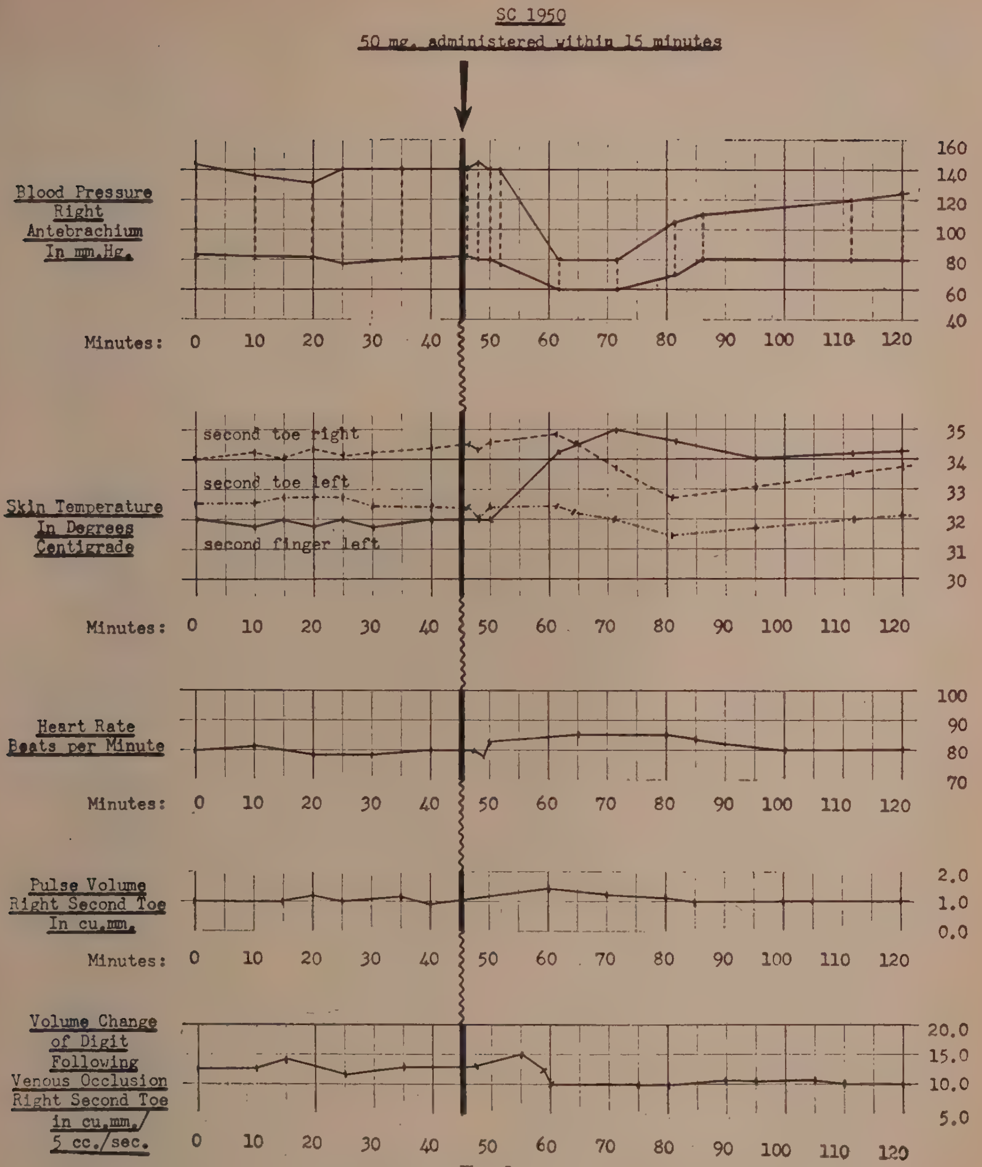

Fig. 3.

Effect of 50 mg SC 1950 intravenously, in 55-year-old male with arteriosclerosis obliterans and polycythemia. Pain in calves upon walking 2 blocks. Hypotensive effect lasted in excess of 55 min. Slight decline in skin temp. of toes may be due to shunting away of blood from diseased lower extremities to more normal parts of body, *i.e.*, second left finger, in which skin temp. was elevated. Heart rate increased slightly, perhaps due to blocking of para-sympathetic nervous system. Pulse vol. changed insignificantly probably on account of arteriosclerotic obliterative disease of peripheral arteries. Vol. change following venous occlusion (relative toe flow) only momentarily higher, then declined somewhat, due to shunting away of blood.

peripheral arterial disease the digital pulse volume increased only insignificantly. (Fig. 3).

Oscillometric readings recorded at the brachium were carried out in a group of 10 individuals composed of normal subjects as well as of patients with peripheral arterial disease. Pulsations in the arteries declined from an average of 7 units (3 to 12) before injection to 3 units (1 to 7.5) ten minutes after administration of the drug. As in 2 patients marked lowering of the height of pulsations was not accompanied by any significant increase in blood flow or skin temperature, this finding will most likely have to be traced to decreased cardiac output.

*Effect of SC 1950 on Digital Blood Flow and Skin Temperatures.* Administration of SC 1950 in normal individuals led to a marked increase in the blood flow to the toe. In a specific case (Fig. 1) the relative toe flow rose from 3.0 cu mm/5 cc per sec. to 25.0 cu mm, as compared with a value of 20.0 cu mm, obtained after release of 5 minutes arterial occlusion above the knee. The skin temperature rose 7°C in each toe. In the same subject body heating, using 2 woolen blankets as well as 2 electric pads for a period of 40 minutes, resulted in an increase in digital flow amounting to 30.0 cu mm/5 cc per sec. In patients with mild peripheral arterial disease, an increase in blood flow to the extremities as well as a rise in the skin temperature was observed upon intravenous instillation of the drug, but the readings always remained decidedly below normal levels. For instance, in a case of early thromboangiitis obliterans of the left leg (Fig. 2) flow to the left toe increased following injection from 2.0 cu mm/5 cc per sec. to 4.5 cu mm. Five minutes after release of arterial occlusion the toe flow reached 5.0 cu mm/5 cc per sec. Under the influence of SC 1950 the skin temperature of the left second toe rose from 26.0° to 32.8°C, a value well below normal, but very similar to the reading of 33.2°C obtained after 40 minutes of body heating. In the presence of advanced peripheral vascular disease neither the digital blood flow nor skin temperature was significantly influenced by administration of SC 1950. A patient with arteriosclerosis obliterans and polycythemia, for instance, (Fig. 3) showed a rise in peripheral flow from 12.0 cu mm to 16.0 cu mm/5 cc per sec., while following release of 5 minutes arterial occlusion it amounted to 9.0 cu mm. Skin temperature of the toes increased only slightly in the right second toe from 34.0° to 34.8°C.

*Vasomotor Reactions and Side Effects.* SC 1950 was furthermore studied as to its possible influence upon the variations in the amplitude of pulse waves as well as in the volume of the digit normally produced through respiration. The 5 normal subjects were examined at 2 minute intervals before and 15 minutes after injection of the drug. Before injection all 5 subjects presented upon sharp thoracic inspiration the normal decrease in the amplitude of pulse waves as well as in digital volume. Following injection of SC 1950 a definitely changed pattern could be observed. Either no reaction whatever appeared upon inspiration, or an increase in the volume of the digit occurred as a paradoxical response. Both effects were taken to indicate that satisfactory blocking of the sympathetic nervous system had taken place.

Side reactions were relatively common and occurred in one-fourth of the 26 subjects. These responses included nasal obstruction, sighing inspiration, weakness, nausea, dizziness, sleepiness, restlessness, angina, dry mouth, swelling of the throat, heavy eyelids, visual disturbance, and thick speech. When SC 1950 was administered rectally as a retention enema in a dose of 25 mg per kg of body weight, the same changes in blood pressure as following intravenous injection were observed, but there occurred also the identical side reactions.

*Summary and conclusions.* SC 1950 has a profound effect on the peripheral circulation. A fall of blood pressure is observed in normal individuals as well as in patients with peripheral vascular disease, but in the latter group this response is subject to considerable variations. The pulse rate is frequently elevated, and considerable variations occur following intravenous injection of SC 1950. The pulse volume of the digits shows a marked increase in normal individuals, is somewhat less in patients with moderate peripheral arterial disease, and varies only insignificantly in patients with advanced organic peripheral arterial disease. In normal individuals the skin temperature of the digits rises sharply, often as much as 8°C, while little change is observed in patients with advanced peripheral disease. Digital blood flow in normal individuals shows a marked increase, while it rises only slightly in patients with moderate or advanced peripheral arterial disease. In all groups the fall in systolic pressure is greater than that in diastolic pressure. From these observations it may be concluded that SC 1950 is an effective spasmolytic agent, capable of increasing the blood flow to the periphery, but beset by a number of side reactions upon intravenous as well as rectal administration.

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# Adsorption, Distribution and Excretion of Tripelennamine (Pyribenzamine). (17702)

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Despite the wide therapeutic usage of tripelennamine (Pyribenzamine), little is known in regard to the physiological disposition of this antihistaminic agent. The urinary excretion of tripelennamine after oral administration has been reported to be about 10%(1), 15.8-25.5%(2), and greater than 85%(3). So far as we have been able to ascertain, the tissue distribution of this compound has not been reported. Our interest in general in the metabolism of basic amines of therapeutic interest led us to investigate the absorption, distribution, and excretion of tripelennamine.

*Procedure and results. Estimation of tripelennamine.* This was accomplished by applying a general procedure for organic bases described by Brodie and Udenfriend(4,5). The method is based upon the extraction of the base from tissue brei with an organic solvent and then reacting the compound with methyl orange to form a colored complex which is soluble in the solvent and can be measured photometrically. Technical grade benzene was found to be a suitable solvent for quantitatively extracting tripelennamine from tissues. Low blanks (optical density less than 0.015) were obtained for most tissues.‡ A high degree of specificity for the method was attained by washing the benzene extract of tissue with 0.5 M phosphate buffer pH 7. This procedure resulted in the removal of a small amount of interfering material, presumably metabolic products of tripelennamine, which reacted as the parent compound.

To illustrate the specificity of the procedure the distribution ratios of tripelennamine between benzene and water at various pH values were compared with those of apparent tripelennamine recovered from tissue minces of rats given the compound. The results, as shown in Table I, indicate that the method is highly specific since the distribution ratios of the recovered drug are in good agreement with those of a known sample of tripelennamine.

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† Present address: Smith, Kline, and French Laboratories, Philadelphia, Pa.

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‡ Technical benzene on standing may give higher brain blanks and must be checked at frequent intervals for such changes. Also, brain blanks vary with different samples of technical benzene.

TABLE I.

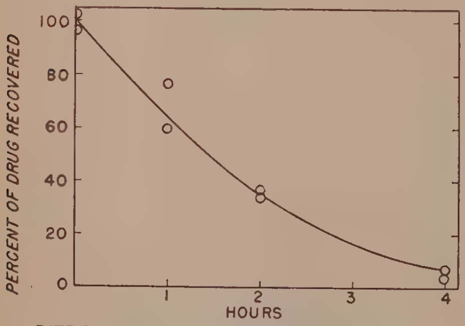
Distribution Ratios of Tripelennamine Remaining in Benzene to Total Drug (Added or Recovered) After Equilibration with Various Buffers.

| | Buffer pH | | | | | |
|---|---|---|---|---|---|---|
| Sample description | 4 | 5 | 6 | 7 | 9 | 14 |
| Added to water | 8 | 40 | 90 | 101 | 104 | — |
| Recovered from liver | 11 | 42 | 85 | 101 | 99 | 103 |
| ” ” kidneys | 12 | 43 | 83 | 95 | 96 | — |
| ” ” brain | 9 | 36 | 90 | 108 | 101 | — |
| ” ” lungs | 6 | 34 | 91 | 104 | 100 | — |
| ” ” spleen | 10 | 41 | 91 | 102 | 101 | — |
| ” ” heart | 10* | 34 | 88 | 103 | 107 | — |
| ” ” muscle | 22* | 41 | 88 | 98 | 106 | — |

* Levels too low for accurate determination.

The likelihood of two substances having the same distribution ratios under such rigidly defined physical conditions is rather remote(4).

*Rate of absorption.* The rate of oral absorption of tripelennamine was studied on 8 rats. After administering 100 mg/kg of the compound by stomach tube, 2 rats were sacrificed at 0, 1, 2 and 4 hours later. Their entire gastro-intestinal tracts were removed, diluted to 500 cc, thoroughly minced, and 1 cc aliquots analyzed for tripelennamine. The results as shown in Fig. 1 suggest that tripelennamine is rapidly and completely absorbed. Excretion of the unchanged compound by the gastro-intestinal tract appears to be relatively minor. To rule out that the rapid disappearance of the compound may be due in part to its destruction in the enteric tract, 2 rats were given a known amount of the compound by stomach tube and sacrificed shortly thereafter. Their entire gastro-intestinal tracts plus contents were removed, suspended in Ringer and incubated for 2 hours. On analyzing for tripelennamine in the usual manner, no alteration in its concentration was noted.



RATE OF DISAPPEARNCE OF PYRIBENZAMINE FROM THE GASTRO-INTESTINAL TRACT AFTER ORAL ADMINISTRATION.

Fig. 1.

*Tissue Distribution of Tripelennamine.* Studies were made chiefly on rats. The first group of animals were given 50 mg/kg of tripelennamine hydrochloride intraperitoneally and were sacrificed at various time intervals. Various organs were removed, minced and analyzed for tripelennamine. The results in Table II indicate that tripelennamine rapidly leaves the blood and localizes in tissues. Considerable variation in tissue levels after intraperitoneal administration of the compound is apparent from the results. It is of interest to note that highest concentrations were found in the lungs. High levels of the agent were also present in the liver, kidneys and the spleen. Brain also contained appreciable quantities of tripelennamine and low levels of the compound were detected in the blood, muscle and heart. That extensive accumulation of tripelennamine does not occur is shown by the fact that the drug was not detectable in tissue 24 hours after its repeated administration for several days. Oral administration resulted in elevated levels in the liver (Table III). Studies made on a guinea pig, gave a distribution pattern similar to that obtained with rats and the localization of tripelennamine in the lungs appeared even greater.

It should be pointed out that in order to increase the accuracy of measuring tripelennamine in tissues, the dose of the drug administered was larger than that usually necessary to induce pharmacologic effects. However, the results resemble somewhat those re-

TABLE II.
Distribution of Tripelennamine in Animals after Intraperitoneal Administration.

| Dose schedule | Time sacrificed, hr | mg/kg fresh tissue | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Brain | Lungs | Liver | Kidneys | Spleen | Heart | Muscle | Blood |
| *Rats* | | | | | | | | | |
| 50 mg/kg | 1 | 27 | 43 | 37 | 31 | 83 | 7 | 6 | 3 |
| | 1 | 18 | 38 | 25 | 29 | 11 | 5 | 3 | 2 |
| | 1 | 15 | 63 | 33 | 30 | 14 | 5 | 4 | 2 |
| 50 mg/kg | 2 | 5 | 24 | 8 | 12 | 5 | 5 | 4 | — |
| 20 mg/kg 2 × daily | 24 | <2 | <2 | <2 | <2 | <2 | <2 | <2 | <2 |
| 4 days | 24 | <2 | <2 | <2 | <2 | <2 | <2 | <2 | <2 |
| 30 mg/kg 3 × daily | 24 | <2 | 2 | <2 | <2 | 4 | <2 | <2 | <2 |
| 4 days | 24 | 2 | <2 | <2 | <2 | <2 | <2 | <2 | <2 |
| 50 mg/kg 2 × within 60 min.* | all dead 10′ after 2nd inj. | 66 | 75 | 187 | 187 | 187 | 27 | 22 | 9 |
| Same | all dead 30′ after 2nd inj. | — | 78 | 105 | 75 | 123 | — | — | 5 |
| 60 mg/kg single inj. | died within 60′ | 35 | 39 | — | — | — | — | — | 25 |
| *Guinea pigs* | | | | | | | | | |
| 50 mg/kg | 1 | 16 | 130 | 14 | 27 | 32 | 10 | — | 2 |
| 100 mg/kg | died within 20′ | 57 | 81 | 270 | 69 | 71 | 54 | — | 12 |
| ” | died within 20′ | 60 | 146 | 270 | 88 | 128 | 37 | — | 23 |

* Pooled organs of 6 rats.

TABLE III.
Distribution of Tripelennamine in Rats 1 Hr after 100 mg/kg Orally.

| mg/kg fresh tissue | | | | | | | | % total dose recovered in gastrointestinal tract + contents |
|---|---|---|---|---|---|---|---|---|
| Brain | Lungs | Liver | Kidney | Spleen | Heart | Muscle | Blood | |
| 7 | 8 | 18 | 7 | <2 | <2 | <2 | <2 | 77 |
| — | 4 | 34 | 9 | 6 | <2 | <2 | <2 | 61 |

ported for another antihistamine agent, diphenhydramine (Benadryl) by Glazko and Dill(6) and our previous findings(7,8) on other basic amines (Demerol, and d,l methadone).

Further evidence that tripelennamine rapidly leaves the blood and concentrates in tissue is indicated in experiments following single and repeated intravenous injections of the drug into the saphenous vein of unanesthetized rats. Although the intravenous $LD_{50}$ of tripelennamine in rats is reported to be 12 mg/kg(9), it was found possible to inject

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6. Glazko, A. J., and Dill, W. A., *J. Biol. Chem.*, 1949, v179, 403.

7. Leong Way, E., Gimble, A. I., McKelway, W. P., Ross, H., Sung, C. Y., and Ellworth, H., *J. Pharmacol.*, 1949, v96, 477.

8. Leong Way, E., Sung, C. Y., and McKelway, W. P., *J. Pharmacol.*, 1949, v97, 222.

9. Mayer, R. L., Hays, H. W., Brousseau, D., Rennick, B., and Yonkman, P. P., *J. Lab. Clin. Med.*, 1946, v31, 749.

TABLE IV.
Distribution of Tripelennamine in Rats after Intravenous Administration.

| Dose, mg/kg | Schedule | Time sacrificed | mg/kg fresh tissues | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Brain | Lungs | Liver | Kidneys | Spleen | Heart | Muscle | Blood |
| 15 | Single inj. | Died 3′ | 152 | 91 | 6 | 5 | 5 | 122 | 20 | 6 |
| 15 | ” | Died 3′ | 85 | 66 | — | — | — | — | — | 16 |
| 7.5 | ” | *3′ | 36 | 51 | <2 | 2 | 6 | 15 | 2 | 3 |
| 5 | ” | 3′ | 20 | 61 | — | — | — | 10 | — | <2 |
| | Repeated inj.† | | | | | | | | | |
| 40 | in 80′ | 30′ | 15 | 16 | 8 | 18 | 18 | 2 | 3 | <2 |
| 40 | 40′ | 30′ | 26 | 36 | 12 | 25 | 22 | 6 | 9 | 11 |
| 35 | 30′ | 30′ | 38 | 67 | 21 | 49 | 24 | 9 | 9 | — |
| 20 | 45′ | 15′ | 24 | 27 | 10 | 20 | 14 | 3 | 3 | 2 |
| 30 | 25′ | Died | 38 | 62 | — | — | — | — | — | 3 |
| 20 | 15′ | ” | 49 | 73 | 10 | — | — | — | — | 14 |
| 50 | 35′ | ” | 55 | 88 | 17 | 92 | 37 | 23 | 14 | 10 |

* Convulsing when sacrificed.
† 5-10 mg fractions at 5-10′ intervals.

the animal with as much as 40 mg/kg by this route when the dosage was administered over a 30-40 minute period in 5-10 mg/kg fractions. On sacrificing these animals 30 minutes after the last injection and analyzing their tissues for tripelennamine, lung levels were again found to be highest. Brain, kidneys and spleen levels were almost as high, and liver concentration, somewhat lower (Table IV). The reversal in order of brain and liver as to the amount of tripelennamine present was the chief difference in tissue distribution found between intravenous and intraperitoneal administration of the compound.

Animals which did not survive a given tripelennamine dosage were analyzed immediately upon cessation of respiration for tissue levels of the compound. The lung and brain levels in the animals which died from intravenous administration were high and liver levels low. In contrast animals which died after intraperitoneal administration of tripelennamine had exceedingly high concentrations of the drug in the liver, spleen and kidneys, but the levels in the brain, lung and heart were approximately comparable to the figures obtained after intravenous administration.

The concentration of tripelennamine in the brain appears to be related to its toxic effects. Four animals which were sacrificed during or shortly after having convulsive seizures had tripelennamine brain levels ranging from 24-38 mg/kg. Of the 4 animals having a brain level between 35-38 mg/kg, 2 died and the other 2 experienced severe convulsions before they were sacrificed. The brain levels of 5 other rats which died were over 40 mg/kg (range 45-152 mg/kg). Also the level from the pooled brains of 6 rats which died from intraperitoneal administration was 66 mg/kg.

*Urinary Excretion of Tripelennamine.* In 4 humans (3 males and 1 female) after 50-100 mg of tripelennamine orally, less than 1% was recoverable in the urine after 15-24 hours. This is considerably less than the values (15.8-22.5%) reported by McGavack, *et al.* who also used the methyl orange technic and is probably attributable to the fact that they used much higher doses of tripelennamine (400 mg). Perlman(1) reported results similar to ours and indicated also that about 10% is excreted in the urine in a conjugated form which can by hydrolyzed in alkali to tripelennamine. In partial confirmation of his findings, we found that on subjecting the urine of individuals receiving tripelennamine to alkaline hydrolysis, the amount of methyl orange reactants recovered was increased about 8 fold. The high urinary excretion values (86-91%) reported by Jones and Brady(3) may be attributed to the fact that they were measuring essentially the metabolic products of tripelennamine.

In 4 rats which were given 50 mg/kg of tripelennamine intraperitoneally, less than 0.5% was present in the urine after 5 hours and less than 2% after 24 hours. Since our earlier studies established that storage of the compound was not prolonged and its excretion via gastro-intestinal tract was negligible, it is concluded that the compound is to all practical purposes completely metabolized in the body.

The low urinary excretion of tripelennamine is not unlike results we have found with other antihistamines, diphenhydramine (Benadryl), phenindamine (Thephorin) and pyranisamine (Neo-antergan), which are excreted in the urine only to a minor extent after oral administration(10,11).

*Role of the liver in the metabolism of tripelennamine.* Preliminary studies *in vitro* and *in vivo* indicate that the liver is important for metabolizing tripelennamine. In studies with tissue slices of various organs, the liver was found to be by far most active for altering tripelennamine. Less than 10% of tripelennamine was recovered after aerobic incubation of 20 μg of the compound for 2 hours at 38° with 200 mg of liver slices suspended in Krebs-Ringer solution. In 2 rats which were partially hepatectomized, severe convulsions were noted and one animal died after the administration of 40 mg/kg of tripelennamine intraperitoneally, whereas this did not occur when 2 laparotomized rats were given the same dose. Also, in our distribution studies with normal animals, convulsions were not noted after 50 mg/kg of tripelennamine.

*Summary.* Tripelennamine (Pyribenzamine) was estimated by the Brodie methyl orange technic which was modified to give a high degree of specificity. The compound is rapidly and completely absorbed by rats following oral administration. It rapidly leaves the blood and localizes in all tissues especially lungs. Brain, kidneys, spleen, liver and heart all may concentrate appreciable amounts, the level of tripelennamine present in each organ being somewhat dependent upon its mode of administration. High brain levels of tripelennamine were consistently noted with lethal doses of the compound. Prolonged storage of the compound does not occur and only small amounts are excreted via the urine and feces. It is concluded that the compound is almost completely metabolized in the body. The liver subsequently was found to be the most active organ for metabolizing tripelennamine.

We are grateful to the American Allergy Foundation for generously supporting our studies and to Dr. Paul K. Smith, Dr. D. L. Howie, Mr. C. Y. Sung, and Mr. J. R. Parikh for their cooperation.

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10. Leong Way, E., Overman, J. R., and Howie, D. L., *Fed. Proc.*, 1947, v6, 382.

11. Unpublished findings.

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# Effect of Feeding Polyoxyethylene Monostearates on Growth Rate and Gross Pathology of Weanling Hamsters.* (17703)

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Considerable interest has developed in studying the effects of ingesting emulsifying agents such as polyoxyethylene derivatives of fatty acids on the assimilation of various lipoidal materials(1). The present study was undertaken to determine the effect of feeding polyoxyethylene monostearate preparations on the growth rate, food efficiency and gross pathology of weanling hamsters.

*Experimental.* Weanling hamsters† (4-6 weeks of age) of mixed sexes were placed in individual cages in an air-conditioned room

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* We are indebted to Mr. Donald Kraybill and Mr. Roy Ring for their assistance in the care of the animals.

1. Anon., *Nutrition Rev.*, 1949, v7, 205.

† Obtained from the Haskins Rabbitry, St. Louis, Mo.

TABLE I.
Composition of Basal Diet.

| Constituent | % | Constituent | mg/100 g |
|---|---|---|---|
| Purified casein | 24.0 | Thiamine hydrochloride | 0.6 |
| Sucrose | 62.4 | Riboflavin | 0.6 |
| Celluflour* | 3.0 | Pyridoxine hydrochloride | 0.6 |
| Salts IV† | 4.0 | Ca pantothenate | 4.0 |
| Fortified cod liver oil‡ | 0.3 | Nicotinic acid | 2.0 |
| 1:20 Liver extract§ | 1.0 | Choline chloride | 100 |
| Cystine | 0.3 | Inositol | 100 |
| Corn oil | 5.0 | Pteroylglutamic acid | 0.2 |
| | | Biotin | 0.01 |
| | | p-aminobenzoic acid | 30 |
| | | 2-methyl-1,4-naphthoquinone | 3.0 |

* Chicago Dietetic Supply Co.

† Hegsted *et al., J. Biol Chem.*, 1941, v138, 459.

‡ 6000 I.U. of vitamin A and 850 I.U. vitamin D per g. Alpha tocopherol was added to supply 2.5 mg per 100 g diet.

§ Wilson Laboratories, Chicago.

maintained at 76°F. All animals were fed a purified diet for a preliminary period of 2 weeks. This diet was designed to be adequate on the basis of information available on the nutrition of the hamster(2,3) and the composition is indicated in Table I. At the end of this 2-week period the animals were randomized among the experimental groups on the basis of weight, gain in weight and sex. Thirty-three animals were selected for each group. The basic diet used during the experimental period of 10 weeks differed from the diet fed during the preliminary period in that the corn oil was reduced from 5% to 1% of the total diet. The appropriate test products were then added at the expense of the sucrose. Two polyoxyethylene monostearate preparations were tested (Sta-soft and Myrj 45) and the results were compared with those obtained with prime steam lard. The following dietary regimens were studied (*ad libitum* unless indicated otherwise): I—5% lard; II—5% Sta-soft; III—5% Myrj; IV—5% lard with the food intake restricted to the amount consumed by the average of Group II or III whichever was the least; V—15% lard; VI—15% Sta-soft; VII—15% Myrj; VIII—15% lard with the food intake restricted to that of Group VI or VII whichever was the least; and Group IX—7½% lard plus 7½% celluflour. The latter diet was included in order to evaluate the effect of feeding a material regarded as essentially inert nutritionally (celluflour) at a level to approximate the non-fatty acid moiety of Sta-soft and Myrj. The lard, Sta-soft and Myrj were kept at all times in a cold room (23°F) and small amounts were removed, melted and incorporated in the diets which were mixed once a week. These diets were then refrigerated at 36°F when not in use. All food remaining in the food cups was discarded at the end of each week and clean food cups provided. The rate of gain, food consumption, mortality, gross pathology of all animals that died during the experiment, and the gross pathology and organ weights of the animals sacrificed at the end of the experiment were determined.

*Results and discussion.* The results for the rates of gain obtained for the ten weeks on experiment are presented in Fig. 1 and 2. The results obtained for the food consumption, food efficiency and mortality are presented in Table II.

It can readily be seen that the ingestion of 5 or 15% Sta-soft or Myrj resulted in slower rates of gain as compared to the ingestion of lard, fed *ad libitum*, and this effect was quite apparent at the end of the second week on experiment. The growth data obtained for Group I (5% lard) and Group II (5% Sta-soft) were analyzed statistically. The F value obtained by an analysis of variance was 19.2 (P = less than 0.01). These groups

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2. Hamilton, J. W., and Hogan, A. G., *J. Nutrition*, 1944, v27, 213.

3. Schweigert, B. S., *Vit. and Hormones*, 1948, v6, 55.

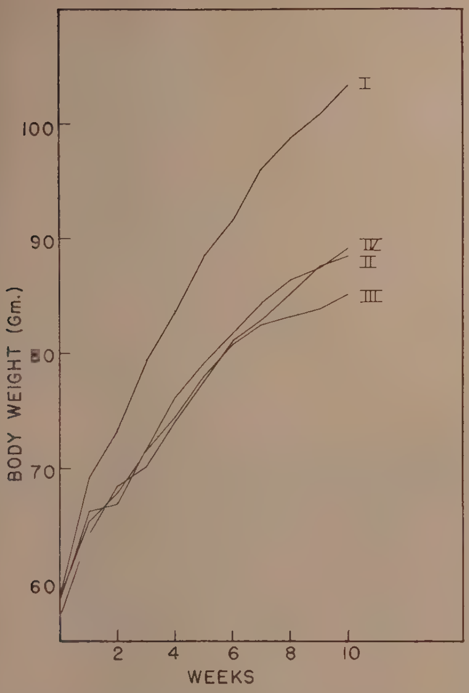

Fig. 1.

Growth curves for animals receiving 5% lard (Curve I), 5% Sta-soft (Curve II), 5% Myrj (Curve III), and 5% lard, food restricted (Curve IV).

were selected since the difference in the mean gains was the least (*i.e.*, 4.4 and 3.1 g per week respectively, as compared to 2.7 g for the animals fed 5% Myrj). The differences in the gains for the groups fed 15% of the test products were even greater. It was concluded, therefore, that the growth rate was significantly reduced and attributable to the ingestion of the Sta-soft and Myrj.

It should be pointed out that the food restriction experiments were not completely successful in that a few of the animals in Group IV did not eat the amount of food determined as the average consumption of Group II or III. Consequently, the group average for Group IV was reduced. It is of interest, however, that even though the food intake was the lowest, the gains were essentially the same as for Group II or III. The superior performance of Group IV as compared to Group II or III is apparent from the food efficiency data (Table I). A separate calculation was made for animals in Group IV that consumed all the ration which was equivalent to the food consumed by Group III, and the average gain per week was 3.5 g (g gained per gram food consumed x 100 = 9.3).

It will also be noted that Groups VI and VII consumed more food than Group V; consequently, the food intake of Group VIII could not be restricted to that for Group VI or VII. Thus Group VIII was essentially an *ad libitum* fed group as most of the animals did not eat the food allotted on the basis of food consumed by Group VI or VII. This is clear from the data on the rate of gain, food consumption and food efficiency, which are essentially the same for Groups V and VIII (15% lard). The group receiving 7½% lard and 7½% celluflour made excellent gains; in fact the best for any group. The food

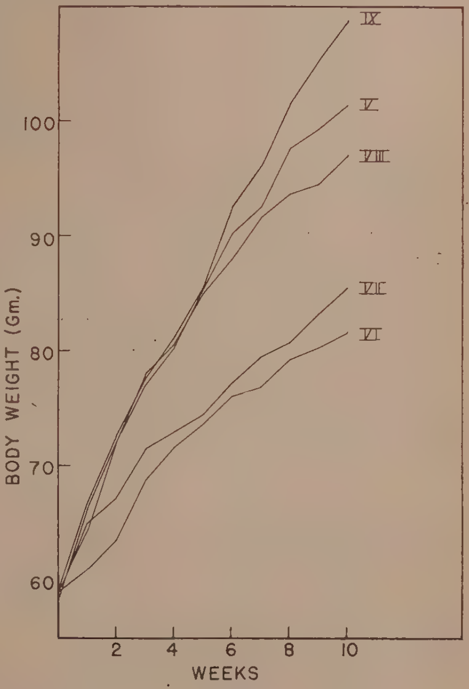

Fig. 2.

Growth curves for animals receiving 15% lard (Curve V), 15% Sta-soft (Curve VI), 15% Myrj (Curve VII), 15% lard, food restricted (Curve VIII), and 7½% lard plus 7½% Celluflour (Curve IX).

TABLE II.
Summary of Data on Rate of Gain, Food Consumption, Food Efficiency, and Mortality.

| Group No. | Test product | Food consumption, g/day | Food efficiency, g gain/g food consumed ×100 | Mortality |
|---|---|---|---|---|
| I | 5% Lard | 5.9 | 10.7 | None |
| II | 5% Sta-soft | 5.7 | 7.3 | 4 |
| III | 5% Myrj | 5.4 | 6.4 | 4 |
| IV | 5% Lard (food restricted) | 5.2 | 8.3 | None |
| V | 15% Lard | 5.0 | 11.4 | 6 |
| VI | 15% Sta-soft | 5.5 | 4.9 | 10 |
| VII | 15% Myrj | 5.5 | 6.0 | 5 |
| VIII | 15% Lard (food restricted) | 5.0 | 10.6 | 2 |
| IX | 7½% Lard<br>7½% Celluflour | 6.2 | 10.4 | 4 |

TABLE III.
Comparison of Organ Weights with Body Weights of Hamsters Fed Various Diets.

| Test product | Wt of organ per 100 g of body weight Liver, g | Kidney, g | Spleen, g | Testes, g |
|---|---|---|---|---|
| 5% Lard | 4.78 | .43 | .14 | .75 |
| 5% Sta-soft | 5.56 | .53 | .14 | .60 |
| 5% Myrj | 4.98 | .52 | .16 | .33 |
| 5% Lard (food restricted) | 5.01 | .46 | .15 | .34 |
| 15% Lard | 4.68 | .41 | .17 | 1.01 |
| 15% Sta-soft | 6.03 | .59 | .16 | .45 |
| 15% Myrj | 5.45 | .55 | .15 | .54 |
| 15% Lard (food restricted) | 4.38 | .43 | .19 | .56 |
| 7½% Lard + 7½% Celluflour | 5.06 | .41 | .17 | .61 |

consumption was also the highest and the food efficiency paralleled the data for Groups I, V and VIII, which received lard without additional celluflour.

Thus the animals in Groups VI and VII compensated at least in part for the apparent caloric inertness of the polyoxyethylene portion of Sta-soft and Myrj and of the additional celluflour in the case of Group IX by consuming more total food. This did not impair animals' performance when receiving celluflour (Group IX), but in spite of the fact that the animals consumed more total food when fed Myrj or Sta-soft, the rates of gain were markedly inferior to those for animals fed lard. It is apparent, therefore, that an inadequate food intake was not the explanation for the inferior performance of the animals fed Myrj or Sta-soft, and that the ingestion of the polyoxyethylene fraction of these products did not show the same results as feeding a substance regarded as essentially inert in caloric value (celluflour).

Although it is difficult to classify the gross pathology noted in a specific sense, it was consistently evident that profound changes in the gastrointestinal tract occurred in all animals fed Myrj or Sta-soft. These changes ranged from a mildly distended intestinal tract with the contents of the lower bowel fluid to a greatly distended intestinal tract with large amounts of gas and fluid present. The cecum was greatly enlarged and for many animals fed 15% Sta-soft or Myrj, the ceca were approximately 4 times the size of those from animals fed lard. Evidence for the presence of blood in the cecal contents was observed for 2 of the animals fed 15% Myrj, indicating hemorrhage. The liver from one animal fed 5% Sta-soft was necrotic and the kidneys from 2 animals fed Sta-soft and 2 animals fed Myrj were grossly abnormal with hemorrhagic areas apparent as well as severe crater-like depressions approximately 1 mm in diameter. Thus, it is clear that not only were differences in the growth rate and efficiency of gain noted, but also differences in gross pathology when hamsters were fed Myrj or Sta-soft as compared to lard.

Evidence of diarrhea and inflammation at the base of the tail was apparent after the animals were fed 5 or 15% Myrj or Sta-soft for 7-10 days. Animals receiving lard were not affected. The mortality was highest in the groups fed Myrj or Sta-soft during the first 4 weeks of the experiment. After this time, the effects seemed to become less severe, the diarrhea or fluid fecal condition was present continuously but did not appear to affect the surviving animals as much as during the early phases.

It will be noted that some animals receiving 15% lard (*ad libitum* and restricted food intake groups) and 7½% lard and 7½% celluflour died during the experiment. The deaths followed no consistent pattern and were observed throughout the test. No good explanation is available for these findings. The rest of the animals showed no effects at the time of the deaths, and although a few showed some evidence of a diarrhea condition, it was of a different nature than observed for the groups fed Myrj or Sta-soft. Nevertheless, no animals receiving 5% lard died, while the corresponding losses in the groups fed 5% Myrj or Sta-soft were 4 and 4 respectively. Considering the groups as a whole, 12 out of a total of 165 hamsters receiving lard died or 7.3% from all causes, while 23 out of a total of 132 receiving 5 or 15% Myrj or Sta-soft failed to survive or 17.4% from all causes. Therefore, although it is quite evident that a higher mortality occurred when Myrj or Sta-soft was fed as compared to lard, the results cannot be considered unequivocal by themselves. They are indicative and strengthen the overall conclusions when considered with the other objective data obtained.

Sections of the small and large intestine, liver and kidney were taken from a representative animal in Groups I, II, III, V, VI and VII at 2, 4, 6, 8, and 10 weeks on experiment for histological examination. Sections were taken from all groups at 6 and 10 weeks. These studies will be presented at a later date.

At the conclusion of the experiment the weights of the liver, kidney, spleen and testes were taken. In these studies, the animals were sacrificed by decapitation, and the organs weighed immediately after dissection. Gross pathological findings were also noted. The cecal contents of two animals receiving 15% Myrj gave positive tests for blood (benzidine test). Representative tests conducted on animals in other groups were negative. The number of animals taken for measurements of organ weights was limited to 18, which included 9 males, since this was the number that survived in Group VI. It should be pointed out that these weights are for the entire liver and spleen, one kidney, and both testes. The data compared on a body weight basis are presented in Table III. No differences were noted in the weights of the livers, kidneys, and spleens for the males and females; consequently, the composite data are presented.

The weights of the liver on a body weight basis are higher for the groups fed 5% Sta-soft (Group II), 15% Sta-soft and 15% Myrj than for the corresponding groups fed lard. Slight increases in the kidney weights were apparent for the groups fed 5% Sta-soft or Myrj and consistent increases for the group fed 15% Myrj or Sta-soft. The data for the groups fed 5% lard were very similar to the data for the animals fed 15% lard. The data obtained for the testes indicate that the animals fed Myrj or Sta-soft had somewhat smaller testes. There was considerable variation in these weights and, further, the limited number of animals studied(9) and lack of agreement in the data for the different groups fed lard do not permit definite conclusions to be made.

It is recognized that there are limitations to evaluating these data only on the basis of weight per animal since the animals fed lard were larger and would be expected to have correspondingly larger organs. Conversely, by calculating the data per 100 g of body weight it is assumed that the ratio of body weight to organ weight is constant, an assumption which is not based on experimental evidence obtained with hamsters. This is quite valid for the animals fed lard, since they approximated 100 grams but the animals fed Myrj or Sta-soft approximated 85 grams in weight. Therefore, these data are regarded as indicative, but not conclusive.

*Summary.* The ingestion of 5 or 15% of polyoxyethylene monostearate preparations (Myrj of Sta-soft) by weanling hamsters significantly reduced the rate of gain as compared to animals fed lard. This was evident after 2 weeks on experiment. There was also a decrease in food efficiency. The retarded gains were not attributable to reduced food intake or to the caloric inertness of the polyoxyethylene moiety of the test products. Marked changes in the intestinal tract and severe diarrhea were also observed when Myrj or Sta-soft was fed. Mortality data, data on organ weights and the gross pathology also indicated that the ingestion of these compounds was deleterious to the hamsters as compared to the data obtained on lard or lard plus celluflour.

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# A Convenient Method for the Determination of Urea in Blood and Urine.

(17704)

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The need for a convenient method of determining urea in blood and urine by direct Nesslerization following the action of urease has been recognized since 1917(1). There is no satisfactory way as yet to avoid cloudy solutions completely. Folin and Wu suggested that urea may be determined in blood and urine by estimating the ammonia formed after heating in acid solution at 150°C(1). Clark and Collip showed that urea is quantitatively converted to ammonia by heating for 10 minutes at this temperature(2), and Leiboff and Kahn devised a special pressure bottle in which the reaction may be conducted in an oil bath at 150°(3). Nevertheless, the method has not been adopted to any appreciable extent, since there was the possibility that ammonia might be formed from other nitrogenous constituents of urine. For more than a year we have obtained satisfactory results for the determination of urea by heating in a modern pressure cooker. The method described below is apparently not affected by other constituents of blood or urine.

*Experimental.* Two household pressure cookers were used in this study. One had a capacity of 4 quarts and a maximum pressure of 15 lb to the square inch (121°C). The other had a capacity of 12 quarts and a maximum pressure of 20 lb (126°C). Using 0.5 ml of 1 M·phosphoric acid to 2 ml of urea solution (0.01 to 0.2 mg·N), 90 minutes are required to decompose urea completely to ammonia at 121°, and 60 minutes are required at 126°. After cooling, the addition of Nessler's solution frequently produces cloudy solutions in less than 10 minutes when protein-free filtrates of whole blood are used. On the other hand, filtrates obtained from oxalated blood plasma or serum with 10 per cent sodium tungstate and 2/3 N·sulfuric acid remain clear for at least 30 minutes after the addition of Nessler's solution. Urine diluted with water also remains perfectly clear. However, urine must first be shaken with permutit to remove ammonium salts before the decomposition of urea. In addition, the concentration of urea may be adjusted to that of blood by diluting the filtered urine, after permutit treatment, according to a table(4),

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1. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, v38, 81.

2. Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1926, v67, 621.

3. Leiboff, S. L., and Kahn, B. S., *J. Biol. Chem.*, 1929, v83, 347.

4. U. S. War Department, *Methods for Laboratory Technicians*, Tech. Manual No. 8-227, p. 107.

TABLE I.
Comparison Between the Values of Urea Nitrogen Determined by the Proposed Method and Those Obtained with Urease Followed by Aeration and Titration.

| | Mg urea N per 100 ml | | | Mg urea N per 100 ml | |
|---|---|---|---|---|---|
| Plasma | Urease method | Proposed method | Urine | Urease method | Proposed method |
| 1 | 13.5 | 14.3 | 1 | 405 | 405 |
| 2 | 15.4 | 15.3 | 2 | 485 | 451 |
| 3 | 15.9 | 16.0 | 3 | 495 | 519 |
| 4 | 16.4 | 16.7 | 4 | 501 | 536 |
| 5 | 18.6 | 18.7 | 5 | 606 | 602 |
| 6 | 19.2 | 19.0 | 6 | 636 | 632 |
| 7 | 22.6 | 22.0 | 7 | 774 | 789 |
| 8 | 24.1 | 24.5 | 8 | 858 | 867 |
| 9 | 25.0 | 25.5 | 9 | 930 | 932 |
| 10 | 39.5 | 40.5 | 10 | 1048 | 1010 |
| 11 | 40.6 | 41.5 | 11 | 1215 | 1240 |
| 12 | 42.5 | 42.4 | 12 | 1360 | 1360 |
| 13 | 52.5 | 53.3 | 13 | 1419 | 1419 |
| 14 | 62.5 | 62.1 | 14 | 1520 | 1490 |
| 15 | 163. | 166. | 15 | 1820 | 1788 |

that is based on the average normal values for urea clearance of Moller, McIntosh, and Van Slyke(5).

The method finally adopted is as follows: Place 2 ml of protein-free filtrate from plasma or serum, or 2 ml of diluted ammonia-free urine, in test tubes graduated at 10 ml. Add 0.5 ml of 1 M phosphoric acid,* and cover the mouths of the tubes with tin foil. Heat for 60 minutes in a pressure cooker at 20 lb pressure or for 90 minutes at 15 lb pressure. After cooling, add 1 ml of 1 N sodium hydroxide and dilute the solutions to 10 ml. Add 1 ml of Koch-McMeekin Nessler's solution, and let stand for 10 minutes. Measure the color in a Klett-Summerson colorimeter with filter 54. A blank tube containing water and acid is heated with each series, and a standard tube, containing 0.03 mg of nitrogen as ammonium sulfate, is prepared for comparison.

The method was tested in a series of samples of plasma and of urine in which urea nitrogen was also determined by incubation with urease, aeration into standard acid, and titration with 0.01 N sodium hydroxide. Table I shows the good agreement of values determined by the proposed method.

*Summary*. An accurate method is described for the convenient determination of urea nitrogen in blood plasma and urine by the estimation of ammonia after heating with acid for 60 minutes in a household pressure cooker at 20 lb pressure or for 90 minutes at 15 lb pressure.

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5. Moller, E., McIntosh, J. F., and Van Slyke, D. D., *J. Clin. Invest.*, 1928, v6, 427.

* The reaction may also be accomplished with 0.5 ml of 1 N hydrochloric or sulfuric acid, but a small yellow-precipitate of hydrated sodium tungstate may appear after heating. The determination is not affected, however, since the precipitate dissolves on the addition of alkali.

# Vascular Reactivity of Rats and Dogs Treated with Desoxycorticosterone Acetate.*† (17705)

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Hypertension and vascular disease can be readily reproduced in rats treated with desoxycorticosterone acetate (DCA) under suitable conditions(1,2). One possible extrarenal mechanism of this hypertension is increase in pressor responsiveness of the vascular tree to agents such as renin, angiotonin and epinephrine. On the other hand, DCA-hypertension of any considerable degree has not been obtained in dogs(3,4). Therefore to assess the contribution of pressor hyperresponsiveness to DCA hypertension we have compared the vascular reactivity of control and DCA-treated rats and dogs.

*Methods.* In the first series of experiments, 21 male albino rats weighing 217 g were divided into 2 groups following unilateral nephrectomy. Animals of Group I received 2 subcutaneous implants of 40 mg pellets of DCA while those of Group II were kept as untreated controls. All the rats were maintained on a high sodium ration by giving 1% saline solution as drinking water. Starting on the 11th day, animals were tested for vascular reactivity to epinephrine, renin and angiotonin. To eliminate any possible variation due to the dilution of these drugs, 1 control was always run simultaneously with 1 or more experimental animals. Following nembutal anesthesia (30 mg per kilo) and heparinization, the carotid artery was cannulated and connected to a mercury manometer. Injections were made into the femoral vein. The dose of each substance was kept constant throughout the experiment and consisted of 0.1 cc (0.125 $\gamma$) of epinephrine, 0.1 cc of renin and 0.2 cc of angiotonin. The stock renin, prepared by Dr. Arda Green, contained 30 mg of protein per cc; 0.1 cc gave a 35 mm Hg rise in a dog under pentobarbital anesthesia. Before use, it was diluted in the proportion of 1 part in 10 physiological saline. Angiotonin, prepared according to the method of Page and Helmer(5) as modified by Plentl and Page(6) contained 10 units per cc and was injected as such.

In the second series of experiments we used 3 mongrel dogs weighing 10 to 20 kg. They were unilaterally nephrectomized, given 1% saline to drink and then were treated with DCA in the form of a water suspension, injected subcutaneously once every 4 days in a dose of 100 mg. Animals were tested before uninephrectomy, after uninephrectomy and after treatment with 500 mg of DCA. During treatment with DCA their daily water intake ranged from 3.5 to 4 liters as saline compared with 500 to 700 cc after operation but before treatment. Vascular reactivity was determined under nembutal anesthesia (35 mg/kg intraperitoneally) by the method described by Page and Taylor(2). The doses of the various drugs were epinephrine 0.1 cc of a 1/20,000 dilution, nor-adrenaline 10 $\gamma$ angiotonin 5 units in 0.5 cc, veratrum alkaloid 29 $\gamma$ in 0.3 cc, sodium azide 125 $\gamma$ in 0.1 cc and tetraethylammonium chloride 5

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* This investigation was supported by a grant from the United States Public Health Service (National Heart Institute).

† Desoxycorticosterone acetate was generously supplied by Dr. E. Henderson of the Schering Corp., and the veratrum alkaloid by Dr. Philip Bates of Coe Chemical Co.

1. Selye, H., Hall, C. E., and Rowley, E. M., *Canad. M. A. J.*, 1943, v49, 88.

2. Masson, G. M. C., Corcoran, A. C., and Page, I. H., *J. Lab. and Clin. Med.*, 1949, v34, 1416.

3. Summers, J. E., *Am. J. Physiol.*, 1948, v154, 119.

4. Davis, W. D., Jr., Segaloff, A., Jacobs, W., and Callohan, J. B., *Proc. Central Soc. Clin. Res.*, 1949, v22, 23.

5. Page, I. H., and Helmer, O. M., *J. Exp. Med.*, 1940, v71, 29.

6. Plentl, A. A., and Page, I. H., *J. Biol. Chem.*, 1945, v158, 49.

TABLE I.
Differences in mm Hg Between Pressor Responses of Control and of DCA-Treated Rats to Epinephrine, Renin, and Angiotonin.

| | 1* | 2* | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Avg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Epinephrine | + 1 | — 4 | + 9 | — 1.5 | +13 | +17.5 | + 4 | + 8 | + 5 | + 5 | +18 | —10 | +5.4 |
| Renin | +10 | — 3 | +18 | — 4 | + 7 | + 3 | +17 | +19 | +21 | —20 | +19 | +14 | +8.4 |
| Angiotonin | — | + 1 | +14 | — 7 | + 6.5 | + 2.5 | +10 | + 4 | + 6 | — 9 | — 3 | +16 | +3.3 |
| Duration of DCA treatment (days) | 8 | 9 | 22 | 32 | 33 | 33 | 34 | 51 | 51 | 52 | 53 | 54 | |
| Blood pressure difference between control and DCA rats | 30 | 20 | 22 | 74 | 60 | 58 | 43 | 56 | 74 | 84 | 48 | 80 | |

* These rats received 0.1 cc of angiotonin.

TABLE II.
Average Vascular Responses of Dogs to Various Drugs.

| | Epinephrine | Nor-adrenalin | Angiotonin | Veratrum alkaloid | Sodium azide | Tetraethyl ammonium |
|---|---|---|---|---|---|---|
| Before nephrectomy | +12 | +54 | +28 | —38 | —40 | —44 |
| After ” | +17 | +54 | +25 | —40 | —48 | —52 |
| ” DCA treatment | +15 | +50 | +22 | —21 | —38 | —34 |

mg/kg body weight (Etamon: Parke, Davis and Co.).

*Results.* Although the same rat gave a relatively constant response to repeated injections of the same dose of epinephrine and angiotonin, there were large variations from animal to animal. In the control group, responses to epinephrine varied from –8 to +15.5 mm Hg, to renin from +11 to +50 mm Hg, and, to angiotonin from +15 to +27 mm Hg. The same group variability occurred in the DCA treated rats: responses to epinephrine varied from +3 to +25 mm Hg, to renin from +15 to +54 mm Hg and to angiotonin from +11 to +34 mm Hg. The response was independent of the duration of treatment and of the degree of hypertension. Since values of the 2 groups overlap each other, we decided that the difference in response to a given drug in the control and simultaneously injected experimental rat would give a clearer picture of the results. In Table I we list these, the duration of treatment and the differences between the blood pressure of control and experimental rats. All the treated rats had an elevated blood pressure; furthermore, all except rats 1, 2 and 3, had vascular lesions of pan- or polyarteritis. These pathologic vascular changes caused by DCA have been described elsewhere in detail(8).

Table I shows, horizontally, the wide variations of response to each drug of treated animals as compared to each other, and, vertically the heterogeneity of responsiveness as between drugs in the same test subjects. Rat No. 12 is hypersensitive to renin and angiotonin and hyposensitive to epinephrine, while the reverse is true in rat No. 10. The blood pressure levels of these 2 rats were the same. Averages in the last column indicate that there is only slight increase in the sensitivity of hypertensive animals.

The results obtained in dogs are summarized in Table II. As expected, uninephrectomy did not have any consistent influence on the vascular response. Therefore, if we accept as normal the variations between the responses

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8. Masson, G. M. C., Hazard, B., Corcoran, A. C., and Page, I. H., *Arch. Path.*, in press.

obtained before and after nephrectomy, one can conclude that DCA did not change the vascular reactivity. It should be pointed out that none of the dogs showed a significant increase in blood pressure as a result of DCA treatment. Blood pressure values obtained regularly by femoral intra-arterial puncture averaged 125 mm Hg (114-148) before and 138 mm Hg (124-150) after treatment. In no instance was there even a consistent trend of blood pressure change as a result of DCA. This is in accord with previous observations that normal dogs are resistant to DCA hypertension(3,4).

It has already been shown that hypertension elicited in dogs by silk perinephritis does not influence the vascular response to various chemical stimuli(7,9). From the present paper the same conclusion applies to DCA treated dogs. Although we found that the average responses to epinephrine, renin and angiotonin were slightly greater in DCA treated than in control rats, it is unlikely that they are significant since many hypertensive rats showed a normal or a subnormal vascular sensitivity. Furthermore, such slight differences would not be sufficient to explain persistent hypertension. The present data do not support the view that part of the pathogenesis of DCA hypertension in the rat can be explained on the basis of a functional hyper-activity of the vascular system(10).

*Summary.* Chronic treatment with DCA of dogs and rats, does not increase significantly vascular response to epinephrine, renin and angiotonin to explain DCA-hypertension on the basis of vascular hypersensitivity.

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## Depression of Tracer Ion Uptake Curve in Rat Erythrocytes Following Total Body X-Irradiation. (17706)

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Radioactive iron given enterally or parenterally has been shown by Hahn and co-workers(1) to be incorporated into the red blood cell as an integral part of the hemoglobin molecule. In addition, an *in vitro* experiment has demonstrated the failure of radio iron introduced into the plasma to exchange with hemoglobin iron of the mature erythrocyte(2). In contrast, more recent work by Walsh *et al.*(3) indicates the ability of circulating reticulocytes to accumulate radio iron *in vitro* to a small extent. Thus it appears that radio iron found in circulating erythrocytes is present principally in cells that have been released from bone marrow following the administration of the tracer dose of iron. Morphologic evidence that the erythropoietic tissue of bone marrow is extremely sensitive has been provided by Bloom and Bloom(4) who found that erythroblasts disappeared from the bone marrow of rats earlier than the myeloblasts following a half lethal dose of X-rays. That this degree of radiosensitivity is compatible with the apparently contradictory findings of normal quantities of circulating erythrocytes is entirely credible when, as has been pointed out by several authors, the longevity of these

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* Post Doctorate AEC Fellow.

1. Hahn, P. F., Bale, W. F., Hettig, R. A., and Whipple, G. H., *Science*, 1940, v92, 131.

2. Hahn, P. F., Bale, W. F., Lawrence, E. O., and Whipple, G. H., *J. Exp. Med.*, 1939, v69, 731.

3. Walsh, R. J., Thomas, E. D., Chow, K. K., Fluharty, R. G., and Finch, C. A., *Science*, 1949, v110, 396.

4. Bloom, M. A., and Bloom, W., *J. Lab. Clin. Med.*, 1947, v32, 654.

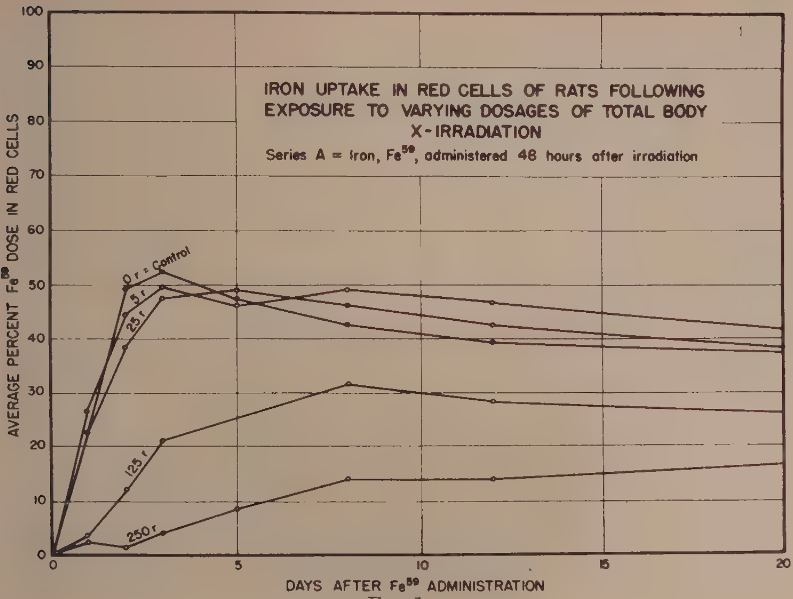

Fig. 1.

cells are considered (5-7).

In view of the ability to demonstrate newly formed red cells with tracer iron and the radiosensitivity of erythropoietic tissue a series of experiments were designed to demonstrate erythroid marrow depression following total body x-irradiation.

*Methods.* Groups consisting of 5 adult female Curtis Dunning rats weighing approximately 200 g were given total body x-irradiation in varying dosages. The radiation was 180 KV X-rays using ½ mm cu and 3 mm aluminum filtration. The animals were radiated individually in a lucite container, and the measurement of the radiation was accomplished with a Victoreen Ionization Chamber inserted in a lucite phantom which approximated the size of the rats used. The animals were divided into 4 series. The control series consisted of one group of 5 rats that received no irradiation. Series A and B consisted of 4 groups that received 5, 25, 125, and 250 roentgens of total body x-irradiation. Series C consisted of 3 groups that received 5, 10, and 25 roentgens. Each animal was given intravenously by tail vein a labeling dose of $Fe^{59}$ in ½ cc of $FeCl_3$ solution which had been buffered to pH 4 with saturated sodium citrate. This dose contained 2 $\gamma$ total iron and approximately .07 microcuries of $Fe^{59}$. In series A the labeling dose was given 2 days after the x-irradiation, in series B one day after, and in series C within 4 hours after the irradiation.

Following the administration of the labeling dose of $Fe^{59}$ ½ cc samples of blood were drawn by means of cardiac puncture. In series A and B samples were drawn at 1, 2, 3, 5, 8, 12 and 25 days. In series C and the control group samples were drawn at 1, 2, 3, 5, 8, 12 and 61 days. The blood samples were heparinized, centrifuged at 2500 RPM for 30 minutes and the hematocrit recorded. Ten milligrams of iron carrier were added to each sample and they were ashed, electroplated, and counted as described in a previous report from this laboratory (8). The calculation of the percent uptake of the administered dose by the red cells was made by cal-

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6. Lawrence, J. S., Dowdy, A. H., and Valentine, W. N., MDDC-836-1946.

7. Dobson, R. L., and Lawrence, J. H., *Ann. Rev. Physiol.*, 1948, v10, 479.

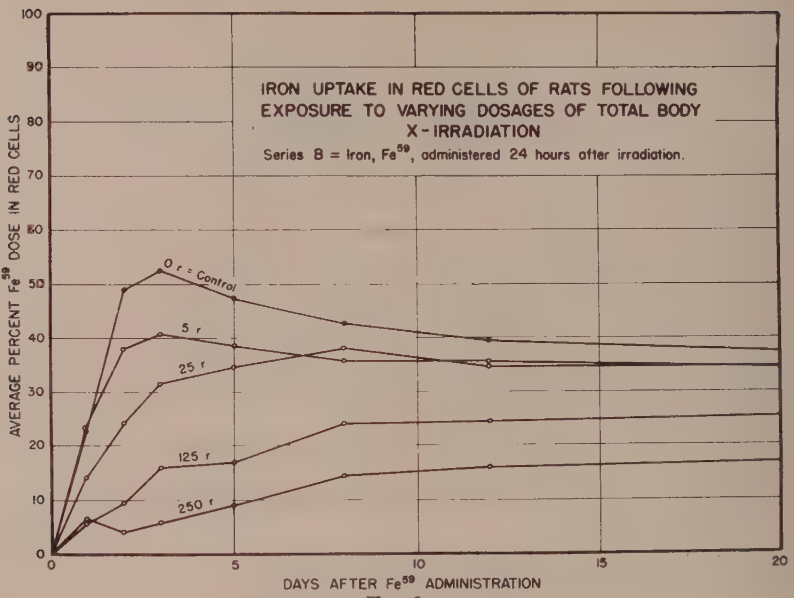

FIG. 2.

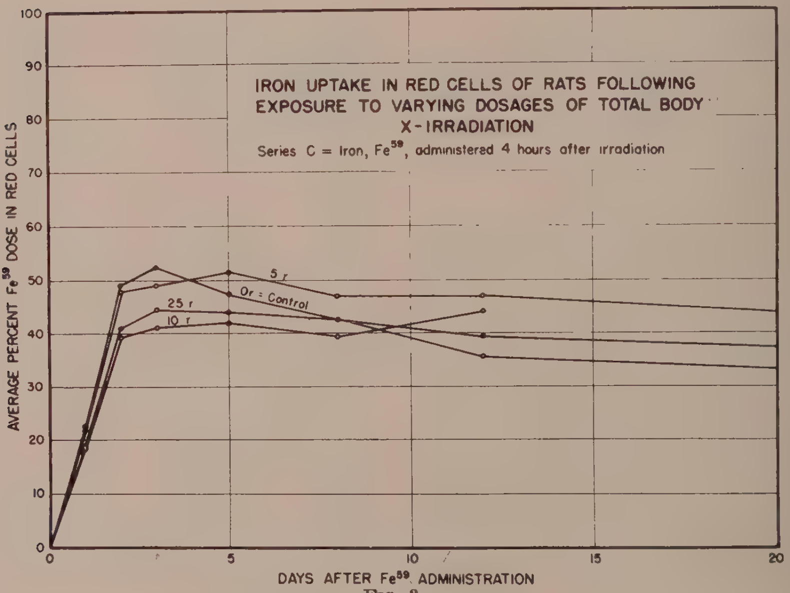

FIG. 3.

culating the blood volume on the basis of 4.59 cc/100 g body weight as given by Berlin *et al.*(8) and multiplying by the hematocrit. Then the total activity of this amount of cells as compared to the administered dose

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8. Berlin, N. I., Huff, R. L., Van Dyke, D. C., and Hennessy, T. G., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 176.

results in the percent uptake as given by the formula:

$$\frac{\text{Animal weight}}{100} \times 4.59 \times \text{Hematocrit} \times$$

$$\frac{\text{CPM/cc of cell sample}}{\text{Total CPM injected}} \times 100 = \%\ \text{dose in red cells}$$

*Results.* The graphs of the results obtained in series A are shown in Fig. 1, series B in Fig. 2, and series C in Fig 3. The control series results are superimposed on each graph. Each point in the graphs represents the average value of the 5 animals in the group except where there was unavoidable mortality due to cardiac puncture.

*Discussion.* The results obtained in these studies show that the radioactive iron red cell uptake curve is a sensitive indicator of x-irradiation damage to the bone marrow. They also confirm the histologic evidence of the sensitivity of erythroid tissue to x-irradiation.

It appears that the greatest depression in erythroid marrow activity occurs at a period of about one day after the irradiation since the groups labeled at this period show a much greater depression at the 5 and 25 roentgen levels.

*Summary.* 1. A method of demonstrating the depression of the erythroid portion of the bone marrow by x-irradiation with the use of $Fe^{59}$ is presented.

2. Graphs of the red cell $Fe^{59}$ uptake curve in rats at various dosage levels of total body x-irradiation and at varying times after irradiation are given.

We wish to extend our sincere appreciation to Doctor John H. Lawrence for his kindly interest and assistance in this work.

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## Assay of Circulating Progesterone by Ultraviolet Spectroscopy. (17707)

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The extraction and analysis of progestin from the blood of mammals has been described by Bloch(1). Although the presence of circulating progestin was corroborated by subsequent investigators, the need for a sensitive and accurate method of assay was obvious. Bloch, utilizing the bio-assay technic of Corner and Allen(2), demonstrated the presence of less than 0.2 μg of progesterone per cc of pooled sow blood. Human pregnancy serum yielded less than 1.0 μg of progesterone per cc. Haskins(3), following the bioassay procedure described by McGinty *et al.*(4), estimated the circulating progestin level in human pregnancy serum at 0.13 μg per cc, expressed as crystalline progesterone. De Allende(5), found the circulating progestin level to vary between 0.06 μg and 2.5 μg per cc of serum in the *Macacus rhesus*, during the menstrual cycle. Hooker and Forbes(6-9), using the method devised by them detected whole blood progesterone concentrations between 4 and 8 μg per cc in rabbits, mice, a monkey and a pregnant woman. The higher progesterone content of blood reported by Hooker and Forbes indicated the possible applicability of ultraviolet analysis of ether extracts of serum for progesterone. The relatively high extinction coefficient of this steroid at 240 mμ renders it readily and accurately measurable in optimum concentrations of 1 to 20 μg per cc solvent (ethanol). Although contaminating substances alter the sensitivity, suitable blanks or controls overcome this difficulty.

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* United States Public Health Fellow in Obstetrics and Gynecology.

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2. Corner, G. W., and Allen, W. M., *Am. J. Physiol.*, 1929, v88, 326.
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4. McGinty, D. A., Anderson, L. P., and McCullough, H. B., *Endocrinology*, 1939, v24, 829.
5. de Allende, I. L. C., Proc. Soc. Exp. Biol. and Med., 1940, v44, 534.
6. Hooker, C. W., and Forbes, T. R., *Endocrinology*, 1947, v41, 158.
7. Forbes, T, R., and Hooker, C. W., *Science*, 1948, v107, 151.
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9. Forbes, T. R., and Hooker, C. W., Proc. Soc. Exp. Biol. and Med., 1949, v70, 682.

A series of experiments were devised in an attempt to demonstrate circulating progesterone by ultraviolet analysis in the blood of pregnant women and in rabbits following the intravenous administration of this substance. Rabbits were also used as test animals in following the blood levels of testosterone and △-4 cholestenone after their intravenous administration. Each of the latter approximates the extinction coefficient of progesterone at 240 mμ.

*Method.* All progesterone determinations were made by ultraviolet analysis. The Beckman spectrophotometer was used with one cm square fused silica cuvettes. All substances analyzed were dissolved in 95% ethanol. Synthetic alpha progesterone with a melting point of 128°C was used. Blood was obtained by venipuncture from women patients whose reproductive status was known. The blood was oxalated and the plasma was expressed by centrifugation. Plasma volumes of between 5 and 10 cc were extracted 3 times with equal volumes of ether and the ether extracts were evaporated to dryness. The residue was dissolved in ethanol upon which ultraviolet analysis was carried out. The plasma extracts of 10 gravid women, 5 postmenopausal women and 14 immediately postpartum women were analyzed in this manner. To determine the recovery of progesterone added to blood plasma, human plasma was obtained from the blood bank and to it crystalline progesterone was added. The plasma thus prepared was extracted and analyzed and compared to control ethereal extracts of plasma. To determine the recovery of progesterone from the blood plasma of living animals, progesterone was administered intravenously to 9 rabbits. Three of this group had been subjected to total hepatectomy under ether anesthesia. Blood was withdrawn by cardiac puncture prior to the injection of the hormone as the control and blank. The progesterone was prepared for intravenous injection into the marginal ear vein of the rabbit in a microcrystalline suspension in 5 cc of normal saline. Cardiac blood was withdrawn at frequent intervals up to 30 minutes after injection of the progesterone suspension. This blood was extracted and analyzed as previously described. Two untreated rabbits were bled as

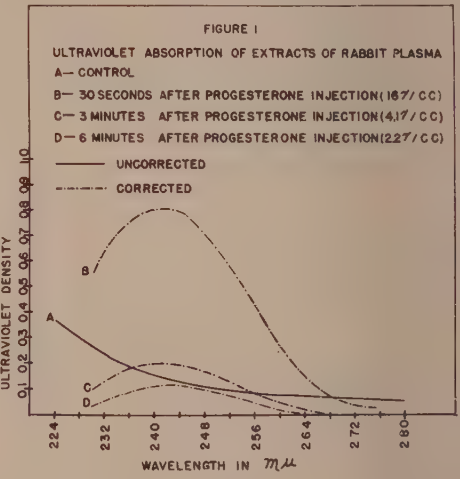


FIGURE I

ULTRAVIOLET ABSORPTION OF EXTRACTS OF RABBIT PLASMA

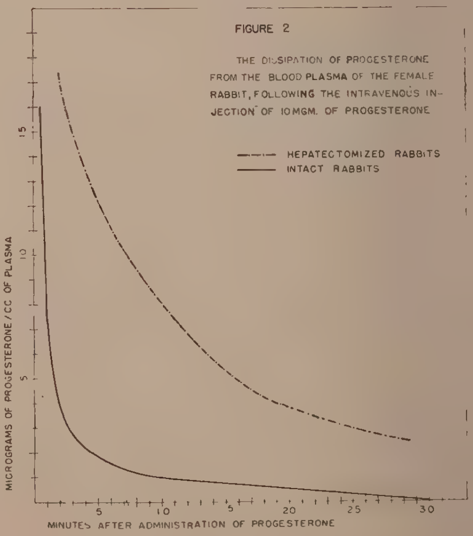


FIGURE 2

THE DISSIPATION OF PROGESTERONE FROM THE BLOOD PLASMA OF THE FEMALE RABBIT, FOLLOWING THE INTRAVENOUS INJECTION OF 10 MGM. OF PROGESTERONE

TABLE I.
**Progesterone Plasma Levels, Expressed as $\mu g$ of Progesterone per cc of Rabbit Plasma, Following the Intravenous Administration of Progesterone. Animals designated A are intact and B are hepatectomized.**

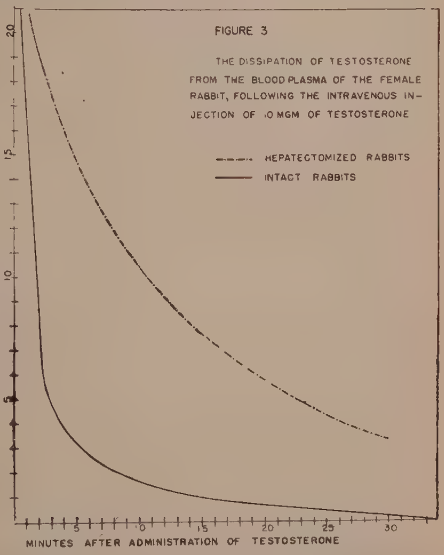
| | Minutes after the injection of progesterone | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/2 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 13 | 20 | 30 |
| A-6 | — | — | 3.7 | — | — | 1.0 | — | 1.1 | — | — | — | — | — |
| A-7 | — | 10.2 | — | 2.7 | — | 2.0 | — | — | 1.1 | — | — | — | — |
| A-8 | — | — | — | 4.0 | — | 2.5 | — | — | 2.3 | — | — | — | — |
| A-9 | — | 6.4 | 4.1 | — | 2.8 | 2.5 | 1.7 | — | — | — | — | — | — |
| A-12 | 16.0 | — | — | 4.1 | — | — | 2.2 | — | — | — | — | — | — |
| A-18 | — | — | — | — | — | — | — | — | — | 0.6 | 0.7 | 0.4 | 0.0 |
| Mean | 16.0 | 8.3 | 3.9 | 3.3 | 2.8 | 2.0 | 2.0 | 1.1 | 1.7 | 0.6 | 0.7 | 0.4 | 0.0 |
| B-10 | — | — | 14.8 | — | — | 15.0 | — | — | — | — | — | 4.4 | — |
| B-15 | — | — | 20.0 | — | — | — | — | — | — | — | — | 3.8 | 2.2 |
| B-17 | — | — | — | — | — | — | — | — | 8.8 | — | — | — | — |
| Mean | — | — | 17.4 | — | — | 15.0 | — | — | 8.8 | — | — | 4.0 | 2.2 |

controls. One rabbit was bled for a period of 3 days following the intramuscular injection of 50 mg of progesterone in sesame oil. Hepatectomized and intact mature virgin rabbits were also given cholestenone and testosterone intravenously. One gravid woman with antepartum eclampsia was injected intravenously with 100 mg of progesterone dissolved in 5 cc of propylene glycol. Blood was obtained for analysis by venipuncture.

*Results.* The analysis of plasma from gravid, postpartum and postmenopausal women failed in any instance to indicate the presence of specific absorption at 240 m$\mu$. Comparative analysis of the absorption curves obtained in these various physiological states revealed no common factor rendering them distinguishable as a group from either of the other groups. In general, the absorption patterns obtained were identical within each group and with those of the other groups.

Patient E. C., to whom 100 mg of progesterone was administered in propylene glycol, revealed no evidence of plasma progesterone prior to injection. At one minute after injection, ultraviolet analysis of the extracts of plasma revealed 3.8 $\mu g$/cc and at 8 minutes after injection, 1.6 $\mu g$ per cc. The sodium pregnandiol glucoronidate urinary excretion was markedly increased two days after injection of progesterone. On the day of injection, the total urinary excretion was found to be 69 mg and 48 hours after injection was 122 mg. Sodium pregnandiol glucoronidate determinations were made after the method of Allen and Viergiver(10).

It was apparent that progesterone injected into the blood stream of the intact rabbits had an exceedingly short existence as ether extractable progesterone. Fig. 1 represents a typical example of the absorption patterns



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10. Allen, W. M., and Viergiver, E. J., *Biol. Chem.*, 1941, v141, 837.

TABLE II.
Testosterone Plasma Levels, Expressed as μg of Testosterone per cc of Rabbit Plasma, Following the Intravenous Administration of Testosterone. Animals designated A are intact and B are hepatectomized.

| | Minutes after the injection of testosterone | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/2 | 1 | 2 | 3 | 4 | 7 | 8 | 10 | 15 | 16 | 25 | 30 |
| A-19 | 20.0 | — | — | 3.6 | — | — | — | — | — | — | — | 0.0 |
| A-21 | — | — | 5.2 | — | — | 2.5 | — | 2.2 | 0.9 | — | — | — |
| A-22 | 21.3 | 14.4 | — | 7.5 | — | — | — | 2.2 | — | — | — | 1.5 |
| Mean | 20.7 | 14.4 | 5.2 | 5.6 | — | 2.5 | — | 2.2 | 0.9 | — | — | 0.8 |
| B-26 | — | 21.8 | — | — | 17.2 | — | 13.4 | — | — | — | — | — |
| B-27 | — | — | — | — | — | — | 11.5 | — | — | 7.5 | — | — |
| B-28 | — | — | — | — | — | — | — | — | — | — | — | 3.7 |
| B-29 | — | — | — | — | — | — | — | 7.4 | 6.3 | — | 2.1 | — |
| Mean | — | 21.8 | — | — | 17.2 | — | 12.5 | 7.4 | 6.3 | 7.5 | 2.1 | 3.7 |

obtained in the experimental animal prior to and after the administration of progesterone. The corrected curves were obtained by using the control analysis as the blank in the spectrophotometer. It will be noted that these corrected curves approach with constant accuracy the absorption pattern of crystalline progesterone dissolved in 95% ethanol. Fig. 2 and Table I illustrate the dissipation of progesterone from the blood plasma of 6 intact and 3 hepatectomized female rabbits. The progesterone level decreases rapidly in the first 6 minutes after injection and then more slowly until at 30 minutes after injection the steroid has disappeared. The hepatectomized group revealed a similar dissipation pattern except that the initial post injection decline was much less precipitous than in the intact animals. Plasma samples from the rabbit treated with intramuscular progesterone in oil at no time exhibited the presence of progesterone.

Those animals to which testosterone was administered, Fig. 3 and Table II, followed the same pattern of decline in circulating hormone level as the progesterone series in both the intact and hepatectomized groups. It will be noted that the initial levels obtained in the testosterone-treated animals were higher than the comparable progesterone-treated animals. This is attributed to the fact that testosterone has a higher solubility in aqueous solvents than does progesterone.

The third series of experimental animals comprise the group to which cholestenone was administered intravenously. At no time in either the intact or the hepatectomized group could a cholestenone plasma level be demonstrated. This was explained on the basis of the minimum solubility of cholestenone of less than 1 μg per cc of water at room temperature. The cholestenone was at no time in solution in the plasma and as particulate matter was rapidly removed from the plasma before even a circulating plasma suspension level could be attained. The recovery of progesterone from plasma to which it had been added was considered to be adequate and varied between 85 and 100%.

*Summary.* Ultraviolet absorption studies of ether extracts of the blood plasma of pregnant women failed to reveal the presence of progesterone. Progesterone was recovered from rabbit plasma by ether extraction and measured by ultraviolet analysis after the intravenous administration of the hormone. In a like manner, testosterone may be recovered from the plasma of rabbits after intravenous administration. Cholestenone plasma levels could not be demonstrated after the intravenous administration of this substance. Testosterone and progesterone have a short life as ether extractable substances in the plasma of intact female rabbits. Hepatectomy decreases significantly the rate of dissipation of ether extractable testosterone and progesterone from the blood plasma of the experimental animal.

Progesterone dissolved in propylene glycol and administered intravenously to a pregnant woman also had a short life as ether extractable steroid. This procedure was followed by a marked rise in urinary sodium pregnandiol glucoronidate excretion.

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# Recovery of the Coxsackie Group of Viruses from Human Sources.
## (17708)

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In 1948 this laboratory reported(1) on the presence of neutralizing antibodies in human sera against the virus of Newcastle Disease (NDV). NDV was not isolated from any of the material examined, and it was shown later (2,3) that the positive neutralization tests were due to a nonspecific heat-labile factor in the sera. Since frozen specimens from the cases described in the previous paper were still available, following the report by Dalldorf and Sickles(4) on the recovery of the Coxsackie virus from human feces(5), this material was re-examined by inoculation into suckling mice. The Coxsackie virus was obtained from both the feces and nasopharyngeal washings of many of these patients. Subsequently, more specimens were examined from various sources, and virus strains have been recovered in material from 97 individuals in 9 different states: Alabama, Colorado, Delaware, Florida, Georgia, Louisiana, Oklahoma, South Dakota and Tennessee. This report presents the results thus far obtained in the study of the viruses isolated.

*Methods.* (a) *Treatment of feces or oral washings.* Fecal and oral specimens were prepared according to the method of Howitt and Barnett(6) for the recovery of poliomyelitis virus. Twenty percent suspensions of feces were made in distilled water. These were spun in an angle centrifuge at 13,000 r.p.m. for 30 minutes in the cold room. One thousand units of penicillin and 10 mg of streptomycin were added for each ml of the supernatant fluid, and the mixtures were left at room temperature for 30 minutes before inoculation of 3-to-5-day-old mice. Each animal received 0.01 ml intracerebrally, 0.03 ml intraperitoneally, and 0.03 ml intramuscularly of the treated feces. The oral washings were inoculated in the same manner, but the antibiotics were reduced to one-half the quantity. (b) *Virus isolations.* The fecal specimens received were kept frozen in the dry ice refrigerator while all other material was stored in the electric freezer at −10°C. The best results were obtained with oral specimens from patients who had gargled with 40 or 50 ml of tryptose phosphate broth. With specimens obtained early in the illness, the virus was found more often in the oral material than in the feces. The inoculation of 3-to-5-day-old mice with positive human material, produced symptoms of marked ataxia, followed by muscle paralysis progressing to complete immobilization, after which death might occur rapidly or be delayed for several days. Virus was present in the infected mice brains, but a larger amount could be harvested by skinning the animals and removing all skeletal muscles. The muscles were prepared as a 10% suspension in buffered saline contain-

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1. Howitt, B. F., Bishop, L. K., and Kissling, R. E., *Am. J. Pub. Health,* 1948, v38, 1263.

2. Howitt, B. F., *J. Immunol.,* 1950, v64, 73.

3. Ginsburg, H. S., and Horsfall, F. L., *J. Exp. Med.,* 1949, v90, 475.

4. Dalldorf, G., and Sickles, G., *Science,* 1948, v108, 61.

5. Gifford, R., and Dalldorf, G., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 589.

6. Howitt, B. F., and Barnett, V. H., *J. Lab. and Clin. Med.,* 1948, v33, 1402.

ing 10% normal rabbit serum and filtered through a Seitz pad. After 2 to 3 passages in mice, muscle filtrates were kept frozen in ampules as the stock virus. (c) *Immune sera.* Immune sera were produced by inoculation of mice, guinea pigs, hamsters, rabbits or monkeys with either the brain or the filtered muscle suspensions. The most effective serum was obtained in about 3 weeks by bi-weekly intravenous injections of rabbits with filtered infected mice muscles. (d) Neutralization tests were performed according to the method in the U. S. Army Laboratory Manual(7) except that the serum-virus mixtures were incubated 20 minutes at 37°C and then inoculated in 0.03 ml amounts intramuscularly into 3-to-5-day-old mice. All sera were inactivated at 56°C for 30 minutes before use. Although the virus strains had $LD_{50}$ titers of between $10^{-6.5}$ and $10^{-8.5}$, many sera gave such high protection that a neutralization index often was not obtained when only 2 or 3 intermediate virus dilutions were used. In tests reported as positive, the serum neutralized at least 1000 $LD_{50}$ doses of virus.

*Characteristics of the virus.* The strain of Coxsackie virus first isolated came from the feces of a child (B.J.A.) in Shreveport, La., submitted for examination in August, 1948, by Dr. C. H. Webb(8). The patient had a severe frontal headache, fever of short duration, but no other symptoms. The specimen was obtained about 4 days after the onset, and upon inoculation 3 months later, yielded a virus that was fatal for 3-to-7-day-old mice and hamsters but not for 14-day-old mice, guinea pigs, rabbits, chickens, embryonated eggs or monkeys (including one 4-day-old monkey). The virus easily passed through a Seitz filter. It could be transmitted by the intracerebral, intraperitoneal, intramuscular, subcutaneous and intranasal routes of inoculation. It was recovered from the blood stream, the brain, cord, lungs, liver, spleen, kidneys, and intestinal contents of the infected mice,

TABLE I.
Summary of Isolations of Coxsackie Viruses from Human Sources.

| State | Oral washings | | Feces | | Blood | | Brain or cord | | Spinal fluid | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. tested | No. positive | No. tested | No. positive | No. tested | No. positive | No. tested | No. positive | No. tested | No. positive |
| Ala.* | 34 | 24 | 16 | 5 | 9 | 8 | 5 | 3 | | |
| Colo. | | | 2 | 2 | | | 1 | 0 | | |
| Del. | 48 | 7 | 31 | 9 | | | | | 20 | 0 |
| Fla. | 3 | 3 | 5 | 5 | 2 | 2 | | | | |
| Ga. | 10 | 6 | 10 | 5 | 2 | 2 | | | | |
| La. | 6 | 2 | 4 | 1 | 1 | 1 | 3 | 2 | | |
| Okla. | 1 | 1 | 4 | 1 | 1 | 1 | | | | |
| S.D. | | | | | | | 1 | 1 | | |
| Tenn. | 16 | 5 | 6 | 1 | 10 | 8 | 4 | 2 | | |
| Totals | 118 | 48<br>(40.6%) | 78 | 29<br>(37.1%) | 25 | 22<br>(88.0%) | 14 | 8<br>(57.1%) | 20 | 0 |

* Virus was recovered from one urine specimen.

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7. Simmons, J. S., and Gentzkow, C. J., Lea & Febiger, 1944, Philadelphia.

8. Webb, C. H., Wolfe, S. G., and Howitt, B. F. Presented at the Annual Session of Am. Med. Assoc., Atlantic City, N. J., June 8, 1949.

and was present in high concentration in the skeletal muscles. Although virus may be found in the brain and cord of infected mice, the histopathological lesions were confined to the muscles as observed by Dalldorf *et al.*(9), Melnick *et al.*(10), and also by Dr. R. E. Kissling of this laboratory. This virus was not neutralized by immune sera for the viruses of MV and Lansing poliomyelitis, MM, encephalomyocarditis, F.A. and GD VII (Theiler's mouse strains), Newcastle disease and herpes.

*Virus isolations from human material.* After the original isolations of the Coxsackie virus from the first few specimens, a systematic study was made of all material remaining from the earlier collections beginning with 1947 through 1949. The Coxsackie virus was isolated from human feces, urine, nasopharyngeal or mouth washings, mouth vesicles, nasal swabs, saliva, sputum, serum, whole blood, cord and brain, but not spinal fluid that had been sent in at various times and from scattered localities in 9 states. All material had been kept frozen at either –10°C or –60°C, and in many instances the virus remained viable after storage for over 2 years at the latter temperature.

Table I gives a summary of 108 isolations of the Coxsackie group of viruses and shows both the geographical location from which the material came and the number of isolations from the different kinds of specimens. While it is believed that this virus is associated with a benign type of infection, it is interesting to note that the virus has been obtained from the nasopharyngeal washings of 1, the serum of 2, and the nervous tissues of 8 fatal cases as shown in Table II. Five of these 10 fatal cases were diagnosed as poliomyelitis. Poliomyelitis and Coxsackie viruses were recovered simultaneously from the spinal cord of one of these patients.

Virus was recovered from either clotted blood or serum of 22 patients. All of the

TABLE II.
Coxsackie Virus Isolations from Fatal Cases.

| Case No. | Age, yr | Location | Date of onset | Date of death | Material tested | Diagnosis | Type of virus isolated |
|---|---|---|---|---|---|---|---|
| 1 | 56 | Ala. | 1/31/49 | 2/14/49 | Brain | Tuberculosis of lungs—encephalomyelitis | 2 |
| 2 | 4 | ,, | 9/ 1/49 | 9/ 5/49 | Spinal cord | Poliomyelitis—Polio virus isolated | 4 |
| 3 | 2 | ,, | 9/ 3/49 | 9/27/49 | ,, ,, | Autopsy findings—negative | 4 |
| 4 | 6 | Ga. | 5/30/49 | 6/ 2/49 | Serum | (?) Mumps encephalitis | 2 |
| 5 | 2 | La. | | 9/ 1/49 | ,, | Poliomyelitis | 2* |
| 6 | 34 | ,, | 8/ 3/49 | 8/11/49 | Brain | Poliomyelitis or encephalomyelitis | 4 |
| 7 | 3 | Tenn. | 7/15/48 | 7/25/48 | Nasal washing | Encephalomyelopathy | 4* |
| | | | | | Brain | | 4* |
| | | | | | Serum | | 4 |
| 8 | 34 | ,, | 11/16/49 | 11/23/49 | Brain stem | Poliomyelitis | |
| 9 | 13 | La. | 10/ 1/49 | 10/18/49 | Brain | Encephalitis (?) | 5 |
| 10 | 20 | S.D. | 12/10/49 | 12/17/49 | Brain | Poliomyelitis encephalitis | 4 |

* Virus of the same type was isolated a second time from the same material.

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9. Dalldorf, G., Sickles, G. M., Plager, H., and Gifford, R., *J. Exp. Med.*, 1949, v89, 567.

10. Melnick, J. L., Shaw, E. W., and Curnen, E. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 344.

blood samples had been kept frozen at –10°C and some were held for over a year. While 14 of the specimens were obtained during the first week of illness, one was taken in 9 and another in 13 days after the onset. The date of onset was unknown on 6 of the patients.

As seen in Table III the Coxsackie virus has been recovered from human material obtained in several outbreaks which, although characterized by diverse clinical pictures, were all suspected of having a viral origin. In children, the picture was usually that of a nonparalytic poliomyelitis or a mild 3-to-5-day fever accompanied by headache and occasionally muscle pains; in adults, it was associated often with an influenza-like syndrome. The largest number of isolations came from material of patients ill during August or September. This observation has been reported also by Curnen, Shaw and Melnick(11). Another peak appeared in March in Montgomery when an outbreak in adults was studied at a military base. Here the patients had an influenza-like disease.

*Differentiation of the Coxsackie viruses into serological types.* Early in these studies it was observed that the antiserum from one strain of virus did not afford protection against two others. When more viruses were isolated, it became apparent that they belonged to several distinct serological types. The presence of a multiplicity of strains for this virus has already been reported by other workers (7,9,12). Through the courtesy of Dr. G. Dalldorf of the New York State Laboratory, antisera were received for viruses which he has designated as Types 1, 2 and 3. By means of both neutralization and complement fixation tests(13), it was found that certain of the strains isolated in this laboratory fell into Types 1 and 2, but none so far into Type 3. In addition, 2 other immunologically distinct viruses have been found that are tentatively called Types 4 and 5 until they can be checked against new strains isolated by other

TABLE III. Outbreaks Associated with Isolations of Coxsackie Virus.

| | | | | | Virus isolations from | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Feces | | Mouth garglings | | Blood | |
| Location | Year | General type of case | Predominant symptoms | Total No. cases tested | No. tested | No. positive | No. tested | No. positive | No. tested | No. positive |
| Wilmington, Del. | | Poliomyelitis-like | Fever, headache, some muscle weakness | 46 | 31 | 9 | 36<br>12 contact nurses | 1<br>6 | | |
| Enterprise, Ala. | 48 | ,, ,, | 3-5-day fever, headache, nausea, vomiting | 18 | 6 | 2 | 5 | 2 | 7 | 6 |
| Shreveport, La. | 48 | Mild fever | 3-day fever, headache, no neurological symptoms | 5 | 4 | 1 | 6 | 2 | | |
| Nashville, Tenn. | 48 | Encephalitis-like or atypical syndromes | Varied symptoms, headache, fever | 17 | 3 | 0 | 13 | 3 | 10 | 8 |
| Montgomery, Ala. | 49 | Influenza-like | Fever, aches, some mouth vesicles | 21 | — | — | 21 | 12 | | |
| Swainsboro, Ga. | 49 | Generalized febrile disease | Fever, headache, vomiting, malaise | 14 | 9 | 4 | 9 | 5 | 1 | 1 |

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11. Curnen, E. C., Shaw, E. W., and Melnick, J. L., *J.A.M.A.*, 1949, v141, 894.

12. Sickles, G. M., and Dalldorf, G., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 30.

13. Howitt, B. F., and Benefield, U. R., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 90.

TABLE IV.
Distribution of Virus Types.

| State | Type 1* | Type 2* | Type 4‡ | Type 5‡ |
|---|---|---|---|---|
| Ala. | 1 | 2 | 14 | 8 |
| Colo. | | | 2 | |
| Del. | 1 | 6 | 3 | |
| Fla. | | | | 7 |
| Ga. | | 1 | 6 | 1 |
| La. | | 3 | 1 | 1 |
| Okla. | | 1 | 2 | |
| S.D. | | | 1 | |
| Tenn. | 1 | 4 | 6 | 1 |
| Total No. typed† | 3 | 17 | 35 | 18 |

* Types 1 and 2: Sickles and Dalldorf (10).

† Each number represents an individual case.

‡ These type numbers are given tentatively pending a cross typing with strains isolated by other workers.

workers. Table IV shows the geographical distribution of the types according to states.

Not all of the viruses isolated have been typed as yet; but thus far, Type 4 has been isolated most frequently, mainly in oral secretions, while Types 5 and 2 are next in prevalence. Type 5 has been recovered most often from Florida and Alabama, while Type 4 predominated in one outbreak in Georgia. So far only Types 2 and 4 have been found in material from fatal cases. Second isolations of virus have been made from 5 of the human tissues, and repeated recoveries have been obtained from the same feces of one patient and the mouth washings of another. Type 4 virus has been found in the brain, oral washings and serum of one person and recovered twice from each of these same specimens, while paired materials (feces and mouth garglings or blood and nasal washings) from 3 individuals have yielded the same type of virus from each pair. The virus could be obtained not only from different tissues or excretions of the same person but also from material taken at different time intervals. Mouth garglings from 4 individuals were positive when obtained 2 weeks after the first isolations. Type 4 virus was isolated 3 times at weekly intervals from one of the patients.

However, there is a doubt as to the exact origin of some of these isolations, because it was found that Type 4 virus could be obtained on 3 occasions from muscle filtrates of suckling mice that had been inoculated with filtered muscles of normal baby mice. These animals had been left for 4 days in the laboratory before they were killed and their muscles passed to other normal mice. Because antibodies for this strain have been found in human sera and because it was first isolated from a human source, it seems more probable that this virus may be disseminated easily in the laboratory rather than that it is normally latent in the mice tissues.

*Neutralization tests against the Coxsackie types of virus.* The different strains of Coxsackie virus were easily distinguished by means of the serum neutralization test because the reactions were clear-cut when appropriate dilutions of virus were used. Most of the strains had an $LD_{50}$ titer between $10^{-6.5}$ and $10^{-8.0}$, so that when the tests were prepared with undiluted standard sera against dilutions of $10^{-4}$ and $10^{-5}$ of the unknown virus, complete protection obtained with only one serum was convincing evidence for the type differentiation. Antibodies against virus Types 1 and 2 were found in human paired sera from one outbreak where both these types of Coxsackie virus had been isolated. Of 13 paired sera tested, 1 was positive for Type 1, 4 for Types 1 and 2, and 6 for Type 2 alone. On 3 occasions only was there a rise in titer between the acute and convalescent sera. Twelve sera of another group showed positive antibodies against 1 or more of the strains, including Types 4 and 5. On 7 occasions antibodies were found in the patient's serum against the same strain of virus isolated from that individual. Positive neutralization tests also were obtained against Type 2 virus in 4 sera from normal individuals. The complement fixation test, using infected muscle as antigen, can also be used effectively for strain differentiation and antibody determination (13). Further serological studies are in progress.

*Discussion.* The Coxsackie virus has been isolated from sporadic cases and from groups of patients involved in diseases with variable and ill-defined clinical pictures such as mild fevers, poliomyelitis-like or influenza-like syndromes, or occasionally encephalitis. The same virus type predominated in 2 localities, while in others the types were mixed. While other workers(8,9,10,11,14) have recovered the Coxsackie viruses from cases without sequelae, it is significant that in this study they were found also in the tissues and secretions of 10 fatal cases, 5 of which were diagnosed as poliomyelitis. Poliomyelitis virus was recovered also from the spinal cord of one of these patients and from the feces of 2 recovered cases. Whether or not the Coxsackie viruses were responsible for death is as yet a matter for conjecture. The frequency of isolation of the Coxsackie viruses from the blood stream is significant not only because of the ease with which the material may be inoculated into suckling mice with little preliminary preparation, but also because the blood is thus a potential source of virus for blood-sucking vectors. Melnick *et al.*(10) have reported on the recovery of the Coxsackie virus from flies taken in nature. However, the evidence at present points to direct person to person transmission because of the ease with which the virus may be demonstrated in the oro-nasopharyngeal passages. The isolation of the virus from the mouth washings of a group of 6 out of 12 nurses (Table III) who were asymptomatic but in contact with patients having illnesses probably due to this virus favors the respiratory route of spread. The discovery of this new group of viruses with its widespread prevalence may require a revision of our epidemiological thought, especially in regard to nonparalytic poliomyelitis and other minor illnesses. The correct interpretation of the association of these viruses with such clinical syndromes has yet to be made, but further studies will no doubt elucidate its true significance.

*Summary.* One hundred eight isolations of the Coxsackie group of viruses have been made from the secretions or tissues of 97 patients from 9 different states with multiple isolations from different kinds of specimens from 6 separate individuals. Virus was recovered a second time from 9 different specimens. The strains obtained may be divided into 4 distinct serological types. Two of them correspond to Types 1 and 2 of Dalldorf; the other 2, tentatively called Types 4 and 5, are antigenically different. The viruses have been recovered from human blood, serum, brain, cord, mouth or nasopharyngeal washings, saliva, sputum, nasal swabs, mouth vesicles, urine and feces with a predominance of Types 4, 5 and 2, respectively. The highest percentage of isolations was from the blood, 22 out of 25 (88.0%) specimens being positive. The virus was found also in material from 10 fatal cases and could be recovered repeatedly from 5 of the same specimens. It was easily isolated from mouth washings during the first week of illness, and on 3 occasions was obtained again from the same patients during the third week after the onset. The largest number of isolations was from material obtained during August and September, with another large group collected in March. Neutralizing antibodies for Types 1 and 2 were found not only in the sera of patients but in those of a few normal individuals as well. The sera of 7 out of 8 individuals showed neutralizing antibodies against their homologous viruses. Type 4 virus has been recovered 3 times from muscle filtrates of normal baby mice and there is evidence that the Coxsackie viruses may be disseminated in the laboratory, thus accounting for some of the isolations.

Grateful acknowledgment is made to VoHammie Barnett and Elizabeth Sims for preparation of the fecal specimens and for performance of the serum neutralization tests. The author is also indebted to the Health Departments of Alabama, Delware, Florida, Georgia, Louisiana, Oklahoma and Tennessee, as well as to Dr. Amos Christie and Dr. J. C. Peterson of Vanderbilt University; Dr. Ralph Paffenbarger and Dr. L. K. Bishop, formerly with the CDC of the U. S. Public Health Service; Dr. G. H. Hauser, Director of the Louisiana State Laboratory; Dr. C. H. Webb of Shreveport, La.; Dr. R. D. Baker of the Alabama Medical School Department of Pathology; Dr. R. L. Worcester, formerly of the Alabama State Health Department; Dr. J. T. Grimes, of Enterprise, Alabama; Dr. E. P. Radford, formerly at Maxwell Air Force Base, Montgomery, Alabama; Dr. J. E. McCroan of the Georgia State Health Department; and various other physicians who have collected or sent in material to the Montgomery Virus Laboratory.

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14. Jaworski, A. J., and West, E. J., *J.A.M.A.*, 1949, v141, 902.

# Reactions of the Isolated Surviving Coronary Artery to Epinephrine, Acetylcholine and Histamine.* (17709)

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During the course of an investigation into the physiologic behavior of the vascular system in rheumatic fever and other disease states, it became necessary to investigate the responses of normal vessels exposed to certain vasoconstrictor and vasodilator drugs. The effects of epinephrine, acetylcholine and histamine were studied in surviving coronary arteries from man, pig, ox and sheep, employing an angioplethysmokymographic technique. The results are presented in this paper.

*Experimental procedure.* The angioplethysmokymographic technique was developed in collaboration with Doctor Jerome T. Syverton(1). In this procedure an isolated surviving blood vessel is perfused and a continuous record is made of the changes in the volume of the blood vessel. A segment of artery (approximately 2 cm in length) is dissected out and mounted with minimum trauma in a plethysmographic chamber as shown in Fig. 1. The plethysmograph is filled with Tyrode's solution and the stopcock is turned so that any variation in the volume of the vessel causes a change in the position of the meniscus of the fluid in the horizontal pipette. The artery is perfused with Tyrode's solution using a perfusion system as illustrated in Fig. 1. The inflow pressure is maintained at 100 cm of water and the outflow pressure at 30 cm of water. The needle at the outlet limits the flow through the artery to 40 cc per minute. Test doses of drugs are injected directly into the perfusate through the modified T-tube in the perfusion system. These test doses flow through the artery in 15 to 20 seconds. The perfusate is oxygenated with 5% carbon dioxide in oxygen, thus providing oxygen for the artery and maintaining the reaction of the perfusate at pH 7.2 to pH 7.4. The perfusion system and plethysmographic chamber are suspended in a constant temperature bath (37.5°C±0.01°C). A photographic record of the volume changes is made at 15 second intervals on a drum camera over a period of several hours. Vasoconstriction is measured by diminution in the fluid volume, and vasodilatation by its increment. Of the two, the former is the more readily obtained measurement; whereas the latter must be dependent upon the initial state of vasomotor activity exhibited by the arterial segment. Dale and Richards(2) have pointed out that it is impossible to demonstrate vasodilatation in vessels already dilated. It follows that in

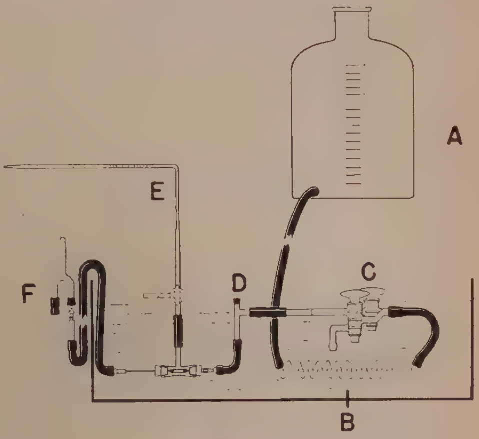

Fig. 1.

Perfusion System. A. Perfusion bottle. B. Warming coil. C. Manifold. D. Modified T-tube for injecting test doses into perfusate. E. Plethysmograph consisting of a 0.2 ml pipette connected to the specimen chamber by a 3-way stopcock. F. Outlet line. B, C, D, E (specimen chamber), and part of F are mounted in a constant temperature bath (37.5°C ± 0.01°C).

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* This investigation was supported by a research grant by the Masonic Foundation for Health and Human Welfare.

1. Smith, D. J., and Syverton, J. T., to be published.

2. Dale, H. H., and Richards, A. N., *J. Physiol.*, 1918, v52, 138.

order to determine vasodilatation, it is necessary that a vessel wall exert some degree of pressure upon the contents of the vessel. There has been some discrepancy between vessels in this respect, particularly in that the human vessels used appeared to possess or to retain this capacity to a lesser degree than did vessels obtained from experimental animals. This may be due to an inherent difference between the coronary arteries of man and those of other species, but may equally well reflect the fact that the human vessels were obtained from subjects who had died of disease while those of the pig, sheep and ox were obtained from slaughtered healthy animals. It will not, in consequence, be possible to draw too close a parallel between the behavior of human coronary arteries and those of experimental animals in respect to vasodilatation. Such differences between the behavior of arteries are for the present considered to be related more to the degree of viability of the individual vessel (or alternatively to the metabolic changes associated with vascular death) than to peculiar differences in their mode of reaction to the same stimulus.

After the artery is mounted in the plethysmographic chamber perfusion is carried out until the artery reaches a constant volume before the test drugs are injected. This usually requires about 2 hours. The baseline obtained is fairly constant for a given artery for a further period of several hours. The volume level assumed by the artery depends in part upon the pressure exerted intrinsically by the arterial wall upon its fluid contents. A satisfactory demonstration of the capacity to dilate of a given specimen was to test the artery with a known vasodilator drug, such as theophylline ethylene-diamine.

*Results.* 1. *Effect of epinephrine.* It has been shown previously(3) that epinephrine causes constriction of most of the peripheral arteries and probably causes dilatation of the coronary arteries(3,4,5,6). The effect of l-epinephrine (synthetic) was tested in 32 coronary arteries from different species in 54 experiments. The results in tabular form are recorded in Table I.

TABLE I.
Reaction of Isolated Coronary Arteries to Epinephrine.

| Species | Man | Pig | Ox | Sheep | Totals |
|---|---|---|---|---|---|
| Vasoconstriction | 18 | 17 | 0 | 1 | 36 |
| Vasodilatation | 1 | 11 | 6 | 0 | 18 |
| Total | 19 | 28 | 6 | 1 | 54 |

TABLE II.
Reaction of Isolated Coronary Arteries to Acetylcholine.

| Species | Man | Pig | Ox | Sheep | Totals |
|---|---|---|---|---|---|
| Vasoconstriction | 17 | 8 | 0 | 1 | 26 |
| Vasodilatation | 0 | 0 | 0 | 0 | 0 |
| Total | 17 | 8 | 0 | 1 | 26 |

TABLE III.
Reaction of Isolated Coronary Arteries to Histamine.

| Species | Man | Pig | Ox | Sheep | Totals |
|---|---|---|---|---|---|
| Vasoconstriction | 27 | 40 | 8 | 1 | 76 |
| Vasodilatation | 0 | 0 | 0 | 0 | 0 |
| Total | 27 | 40 | 8 | 1 | 76 |

2. *Effect of acetylcholine.* Acetylcholine is a dilator of the peripheral arteries(3) but its effect on the coronary arteries has not been precisely demonstrated by previous experiments. Acetylcholine was used 26 times on 18 coronary arteries, with the results shown in Table II.

3. *Effect of histamine.* Best and McHenry (7) have reviewed the data on histamine including the action of histamine upon the coronary arteries. They quote several investigators, using isolated rings and strips of coronary arteries, as having found constriction to be caused by histamine in man, dog and ox. Anrep(5), however, found that histamine produced coronary vasodilatation in perfusion experiments upon the human heart.

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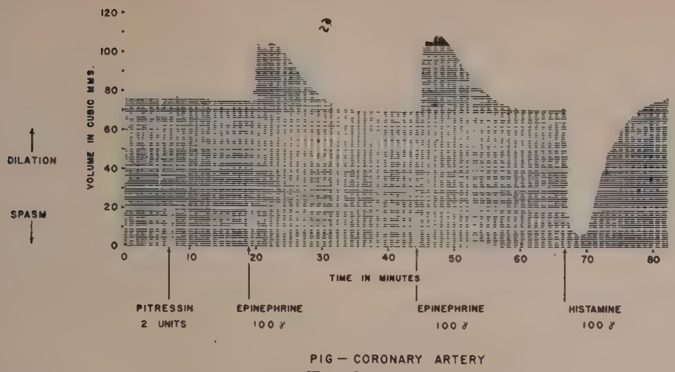

FIG. 2.
Record of the reactions of the coronary artery of a slaughtered healthy pig.

Essex *et al.*(6) demonstrated increased coronary flow following histamine injection in the unanaesthetized dog.

In the present series of experiments 76 observations have been made on 32 coronary arteries. The results are tabulated in Table III.

4. *Sample experiments.* The following are the individual records of 2 typical experiments. The dosages of the drugs employed are noted on Fig. 2 and 3 and throughout the series of experiments varied from 0.1 γ to 100.0 γ. These dosages give concentrations of between 1 part per 100,000 to 1 part per 100,000,000 under the experimental conditions employed.

Exp. 522: Fig. 2. This experiment records the reactions of the coronary artery of a healthy pig. The specimen was mounted 2 hours after the animal had been slaughtered. Pitressin caused no reaction. Epinephrine caused vasodilatation on 2 occasions. Histamine produced vasoconstriction.

Exp. 588: Fig. 3. This coronary artery† was derived from a 23-year-old white female who had succumbed to Hodgkin's Disease. It was removed at autopsy and mounted in the plethysmograph 2 hours postmortem. Again, in these experiments histamine and acetylcholine both produced vasoconstriction. Epinephrine in similar doses produced slight vasoconstriction, much smaller than that produced by histamine and acetylcholine.

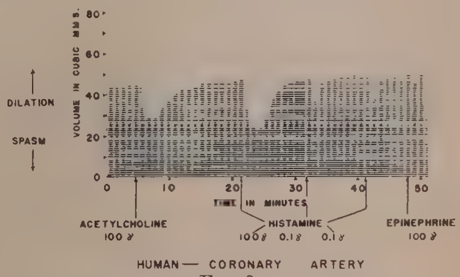

FIG. 3.
Record of the reactions of the coronary artery of a 23-year-old white female who died of Hodgkin's disease.

*Discussion.* The responses of the coronary arteries to epinephrine were of two types and appeared to depend on the degree of vasomotor activity exhibited by the segment under test. Those arteries which were nearly completely dilated (as determined by their failure to respond to known vasodilators) responded to epinephrine by a slight, transient vasoconstriction as in Fig. 3. Those arteries which were not as completely dilated always responded to epinephrine by dilation as in Fig. 2. Certain of the pig arteries were completely dilated at the beginning of the experiment and would show only constriction to epinephrine; later the vessel wall would assume a certain degree of tone and under these conditions epinephrine produced only dilation of the vessel. In these specimens in which both constriction and dilation were recorded the constriction recorded in the fully dilated specimen seemed to correspond closely in size, shape and timing to the notching of the curve of dilation as in Fig. 2. None of the human coronary arteries ever assumed any significant degree of tone. Variations in dosages of test drugs employed varied the magnitude and duration of the reaction produced; it never reversed the response to a given drug (as from constriction to dilation or vice versa). Acetylcholine in varying doses uniformly produced constriction of the coronary arteries examined. Histamine likewise produced only vasoconstriction of the coronary arteries examined; this effect was uniform regardless of variation in the dosage employed (Fig. 3). It is felt that this technic will prove of value in assessing the effect of drugs and hormones upon the coronary and other arteries and that different responses may be obtained in vessels from subjects suffering from different pathological conditions.

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† All human tissues studied were supplied through the Department of Pathology, Rochester School of Medicine and Dentistry. Their cooperation in this study is hereby acknowledged.

*Summary.* 1. The method of employing angioplethysmokymography to evaluate the responses of coronary arteries to several drugs is described.

2. Epinephrine did not produce a uniform response in the coronary arteries examined. The relation of this inconsistency to the state of vasodilation of the specimen artery is discussed.

3. Acetylcholine was found to produce consistent coronary vasoconstriction in man, pig and sheep.

4. Histamine uniformly produces vasconstriction of the coronary arteries of man, pig, ox and sheep.

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## Release of Histamine During Hemolytic Reactions in the Blood of Rabbits.

## (17710)

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It has been shown in different species that histamine is released from the cells of one or several organs during anaphylactic reactions(1-6). Special circumstances pertain in the blood. Most of the histamine contained in blood is held in the white cell layer which separates between the plasma and the red cells when unclotted blood is centrifuged(7,8). This bound or fixed histamine is released by comparatively mild trauma such as clotting or agitation. During anaphylactic reactions in drawn blood, Katz has found that histamine is released from the cells into the plasma(9). In Katz's experiments, rabbits were sensitized by several intravenous injections of egg white. Later when the antigen was added *in vitro* to heparinized samples of the blood the histamine content of the plasma increased 100 to 600% above that of plasma of control samples of the same blood to which no antigen had been added. Studies by Dragstedt and his co-workers(10,11) and Rose and Browne(12) have confirmed and

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11. Gotzl, F. R., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1942, v74, 33.

12. Rose, Bram, and Browne, J. S. L., *J. Immunol.*, 1941, v41, 403.

extended these findings. Dragstedt, Ramirez de Arellano, Lawton and Youmans(13) have demonstrated the liberation of histamine from the blood cells of passively sensitized rabbits when the appropriate antigen was added *in vitro* to the blood. Katz and Cohen(14) extended their observations from rabbits to studies of the human being. They found that histamine was released from the blood cells of patients with ragweed sensitivity when an extract of ragweed pollen was added to the blood *in vitro*. Such a release did not occur when the extract was added to the blood of normal subjects or when extracts of other pollens were added to the blood of patients sensitive to ragweed.

This study was undertaken to determine whether or not hemolytic reactions in drawn blood would release histamine from the cellular elements into the plasma.

*Methods.* Rabbits were used throughout this investigation because the histamine content of their blood is high. This makes it especially suitable for *in vitro* studies. Hemolytic reactions were produced by the addition of sheep erythrocytes to the drawn blood of animals previously sensitized to this antigen. The erythrocytes were obtained by drawing blood from sheep into a solution of sodium citrate to make a final concentration of 0.25% sodium citrate. After centrifugation and discarding the plasma, the red cells were washed by centrifuging three times with a physiologic solution of sodium chloride. The rabbits were sensitized to the washed sheep cells by 4 intravenous injections 1.0 cc of a 50% suspension of the cells given at 4-day intervals. Experiments were conducted no sooner than one week following the final injection of sheep erythrocytes. This method of sensitizing the rabbit to sheep erythrocytes is a modification of that used by Kolmer(15).

To minimize trauma and contact with foreign surfaces, blood was obtained directly from the cut end of a carotid artery. The rabbits were anesthetized with pentobarbital sodium. The carotid artery was dissected free from surrounding tissues in the neck and a small hemostat was placed on branches of the vessel. The artery was then sectioned and the attached hemostat was used to direct the spurt of blood from the artery into a vessel previously coated with paraffin wax. Blood samples were kept in an unclotted state by the addition of heparin.

Histamine analyses were made according to the Code modification of the Barsoum and Gaddum method(16). In the experiments in which plasma histamine was determined, it became apparent that contact of blood even with clean glassware during separation of the plasma was often accompanied by a considerable release of histamine from the formed elements of the blood. Great care was then exercised to avoid undue trauma to the blood by the use of paraffin-coated glassware and by directing the spurting arterial flow down the side of the collecting tube so that no foaming occurred, and by minimizing agitation of the blood. With these precautions only very small quantities of histamine were found in control samples of plasma.

Determinations of antisheep erythrocyte hemolysin titer in the plasma or serum of the rabbit were made according to the method described by Kolmer. In the majority of the experiments, 10 cc samples of rabbit blood were placed in paraffin-coated centrifuge tubes. One cc of physiologic salt solution, or physiologic salt solution in which was suspended washed sheep erythrocytes in a concentration of 50% by volume, were added to equal samples of the blood. After incubation for one hour at 37°C, the samples were centrifuged and the supernatant fluid analyzed for its histamine content. Many of the tests were carried out with duplicate 10 cc samples of blood.

*Results.* Tests were performed on 11 sensitized rabbits. The histamine content of the

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13. Dragstedt, C. A., Ramirez de Arellano, Max, Lawton, A. H., and Youmans, G. P., *J. Immunol.*, 1940, v39, 537.

14. Katz, Gerhard and Cohen, Stanley, *J. A. M. A.*, 1941, v117, 1782.

15. Kolmer, J. A., A Practical Text-book of Infection, Immunity and Biologic Therapy, Ed. 3, Philadelphia, W. B. Saunders Company, 1924, 1210 pp.

16. Code, C. F., *J. Physiol.*, 1937, v89, 257.

TABLE I.

Increase in Histamine Content of Plasma During *in vitro* Hemolytic Reactions in Rabbit Blood.

| | | | *In vitro* study | |
|---|---|---|---|---|
| | | | μg histamine/cc plasma in | |
| Rabbit | Blood hemolysin titer | Min of incubation | Control sample: Blood + 1 cc saline sol. | Test sample: Blood + 1 cc saline sol. containing sheep erythrocytes |
| 1 | * | 60 | .32 | .60 |
| 2 | * | 60 | .67 | 1.28 |
| 3† | 1:5,000 | 60 | .07 | .80 |
| | | | .07 | .70 |
| 4† | 1:10,000 | 60 | .09 | 1.14 |
| | | | .08 | 1.22 |
| 5† | 1:10,000 | Nil | .03 | 1.33 |
| 6† | 1:2,500 | 60 | .13 | .28 |
| | | | .13 | |
| 7† | 1:10,000 | 30 | .20 | 1.25 |
| | | | .33 | 1.14 |
| 8† | 1:50,000 | 30 | .33 | 1.09 |
| | | | .38 | 2.00 |
| 9† | 1:25,000 | 30 | .25 | 1.25 |
| | | | .09 | |
| 10† | 1:50,000 | 30 | .75 | 1.20 |
| | | | .71 | |
| 11† | 1:50,000 | 30 | .13 | 1.02 |
| | | | .11 | 1.13 |

* Hemolysin titer not determined, but extensive hemolysis occurred upon addition of sheep erythrocytes.

† Paraffined glassware used in test.

plasma in which a hemolytic reaction had occurred was consistently increased being usually 2 to 15 times that of control samples (Table I). In one test carried out without incubation, the release of histamine was apparently completed within the time required for separation of the plasma (about 5 minutes, Table I, rabbit 5). A 50% suspension of sheep erythrocytes such as was used to evoke the hemolytic reactions was found to contain less than 0.03 μg of histamine per cubic centimeter. The histamine released during the reaction was therefore not derived from the sheep erythrocytes.

As a means of testing whether or not sheep plasma elements adhering to the erythrocytes were participating in the reactions in rabbit blood the physiologic salt solution used in the final washing of the sheep erythrocytes was tested as an antigen with blood obtained from the sensitized rabbits. Heparinized blood of 2 rabbits sensitized to sheep erythrocytes was divided into a series of equal samples. To 4 of these, physiologic salt solution was added. To another 4, samples of the physiologic salt solution used in the final washing of sheep erythrocytes was added, and to a third group of samples washed sheep erythrocytes were added. All samples were incubated for one hour at 37°C. There was no significant release of histamine into the plasma of the blood to which had been added either pure physiologic salt solution or the physiologic salt solution last used in the washing of the sheep erythrocytes (Table II). As in the other tests, there was, however, a pronounced rise in the plasma histamine in those blood samples to which washed sheep erythrocytes had been added. It is concluded that the release of histamine occurring during the hemolytic reaction was not brought about by elements of sheep plasma adhering to the erythrocytes.

*Comment.* The results obtained indicate that in the rabbit histamine is released from the formed elements of the blood during hemolytic reactions. Loss of histamine from the cells of the blood was not, however, directly checked in this study. It seems unlikely that

TABLE II.
Concentration of Histamine in Plasma of Rabbit Blood After *in vitro* Additions of Sheep Erythrocytes, Saline Solution Washings of the Erythrocytes, and Physiologic Salt.

| | μg histamine/cc plasma of blood to which had been added: | | |
|---|---|---|---|
| Rabbit | 0.5 cc of physiologic salt sol. | 0.5 cc of final washing sol. | 0.5 cc of 50% suspension of washed sheep erythrocytes |
| 12 | .25 | .20 | 1.25 |
| | .09 | .21 | |
| 13 | .13 | .33* | 1.02 |
| | .11 | .36* | 1.13 |

* Blood shaken to effect adequate mixing.

histamine was produced by the reaction and much more probable that its distribution was altered through a liberation from the cells of the blood. If this was the case, the plasma histamine values following the *in vitro* hemolytic reactions were so high that the histamine could not have been derived from the red cells alone but must have come in the main from the elements of the buffy coat. This participation of the white cells and platelets in the hemolytic reaction was further indicated by the reduction in the size of the buffy coat layer in those samples in which hemolytic reactions occurred.

The mechanism by which the hemolytic reaction releases histamine from the formed elements of the blood is not known. A release of histamine into the plasma of drawn blood takes place after such simple trauma as contact of the blood with glass or with fragments of debris or by clotting or shaking of the blood. The release of histamine in the course of the hemolytic reaction is most likely the result of some form of chemical or physicochemical trauma.

The results of this study indicate that release of histamine from blood cells into plasma may contribute to the symptoms observed during hemolytic transfusion reactions.

*Summary.* Rabbits were sensitized by the repeated intravenous injection of washed sheep erythrocytes. After an appropriate period, addition of the washed sheep red cells to the rabbit blood *in vitro* produced prompt hemolysis. This *in vitro* hemolytic reaction in the rabbit blood was always accompanied by an increase in the histamine content of the plasma.

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## The Influence of the Spleen on Hematopoietic Recovery After Irradiation Injury.* (17711)

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It has been reported by Jacobson and associates(1,2) that if the spleens of mice are mobilized surgically and protected with lead during whole body exposure to a dose of 600

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* This investigation was supported (in part) by a research grant from the National Cancer Institute, U.S. Public Health Service; and the Armour Laboratories.

1. Jacobson, L. O., Marks, E. K., Gaston, E., Robson, M., and Zirkle, R. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 740.

2. Jacobson, L. O., Marks, E. K., Robson, M., Gaston, E., and Zirkle, R. E., *J. Lab. Clin. Med.*, 1949, v34, 1538.

TABLE I.
Preparation and Treatment of Animals.

| Group | No. of mice | Preparation of animals | Treatment |
|---|---|---|---|
| I | 25 | None | None |
| II | 35 | Anesthetic (nembutal)<br>Surgical mobilization of spleen | ” |
| III | 105 | Same | 1025 r whole body X radiation including spleen |
| IV | 100 | ” | 1025 r whole body X radiation, spleen lead protected |

r of X radiation, compensatory ectopic blood formation appears in the protected spleens within 24 to 48 hours. Concurrently, a transient leucopenia and thrombocytopenia unassociated with anemia appear in the peripheral blood. An anemia and a severe and more persistent leucopenia and thrombocytopenia invariably occur if the spleen is not lead protected during whole body X radiation with dosages of 600 r or above. Even with a dose of 1025 r whole body X radiation which is 300 roentgens above $LD_{100}$ for intact mice, ectopic blood formation appears rapidly in the lead protected spleen and no anemia and only a modest and transient leucopenia and thrombocytopenia develop. By the third to fourth day after irradiation, the bone marrow of mice exposed to dosages of 600 r and above is largely depleted of free nucleated cells with the exception of a few scattered erythroblasts, plasma cells, and unidentified round cells. Typically the marrow at this stage consists of reticular cells, dilated blood sinuses, hemorrhage, fat, and a homogeneous material which stains slightly blue with hematoxylin-eosin-azure. The $LD_{50}/28$ days for mice which have had lead protection of the surgically mobilized spleen during X irradiation is about 1025 r; the $LD_{50}$ for mice without lead protection of the spleen is about 550 r. Thus lead protection of the spleen increases the $LD_{50}$ by a factor of nearly two. This report relates observations on the regeneration of the hematopoietic tissue of mice exposed to 1025 r with or without lead protection of the spleen. A complete report, including the hematologic data and more extensive illustrations and descriptions of the histopathologic findings, will be published elsewhere.

*Methods.* Four groups of CF-1 female mice†, 10 to 12 weeks of age and weighing approximately 25 g, were prepared as indicated in Table I. Mice in Group I were untreated controls. The mice in Groups II, III, and IV were anesthetized with nembutal (0.072 mg/g), an incision made in the left upper quadrant of the abdomen, and the spleens mobilized through the incision with the main pedicle intact. Group III and Group IV mice were irradiated with 1025 r whole-body X radiation while the spleens were without the abdominal cavity. During irradiation the spleens of Group III were wrapped in gauze and kept moist with physiological saline; whereas, the spleens of Group IV were placed in one-quarter inch thick lead boxes with openings for the splenic pedicle only. Thus the whole body including the spleen of Group III mice was irradiated whereas Group IV mice received whole body irradiation but the spleens were lead shielded during irradiation. The irradiation required about 15 minutes. After irradiation the spleens of Groups II, III, and IV were returned to the abdominal cavity and the incision sutured. The X-rays were generated in a 250 Kv machine operating at 15 ma. A 0.25 mm copper filter was used. The half-value layer of the filtered beam was 1.0 mm. The mice were weighed daily for the first 9 days and every 4 days thereafter. Mice from each

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† CF-1 (Carworth Farms albino with brown, non-agouti background) Homozygous for the recessive genes aa bb cc.

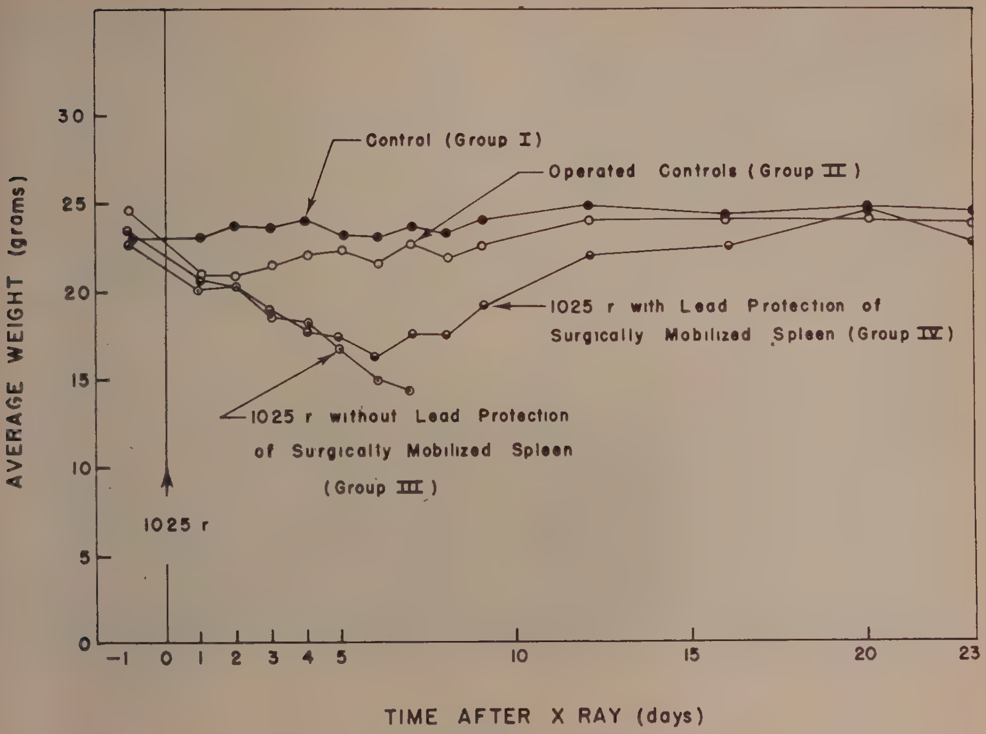

FIG. 1.
The mean weight of control and irradiated mice.

group were killed at daily intervals and tissues fixed in Zenker-formol embedded in nitrocellulose, sectioned at 5 to 8 mu and stained with hematoxylin-eosin-azure.

*Results.* As indicated in Fig. 1, the mean weight of the 2 groups of irradiated mice followed the same pattern of decline. However, all the mice without lead protection of the spleen had died by the ninth day; whereas, the mice which had lead protection of the spleen reached a minimum weight by the sixth day and thereafter rose to approximate the controls by the twentieth day after irradiation. In the mice prepared for histologic study, no regeneration of bone marrow, thymus, or lymph nodes was as yet apparent on the tenth day and only focal areas of regeneration were apparent in the bone marrow on the eleventh day in mice which received 1025 r without lead protection of the spleen; death of all animals in the group precluded study of hematopoietic regeneration in this group beyond this interval. In the mice which received this dose but which had lead protection of the surgically mobilized spleen during irradiation, regeneration of the bone marrow was already apparent at 4 and 6 days and normal in cellularity at 8 days. (Fig. 2) Reconstitution of the lymph nodes in terms of cellularity was also within normal limits by 8 days. The thymi appeared to resume normal cellularity more slowly. In mice exposed to 600 r X radiation with or without lead protection of the spleen, the difference in the hematopoietic recovery rate of the two groups is less striking but nevertheless clear-cut. The bone marrow of rats which have been exposed to dosages of 600 r or 800 r with or without lead protection of the spleen has been examined and demonstrates the same phenomenon(3). Recovery of the irradiated

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3. Jacobson, L. O., Simmons, E. L., Marks, E. K., Robson, M. J., and Gaston, E. O., 1949, unpublished data.

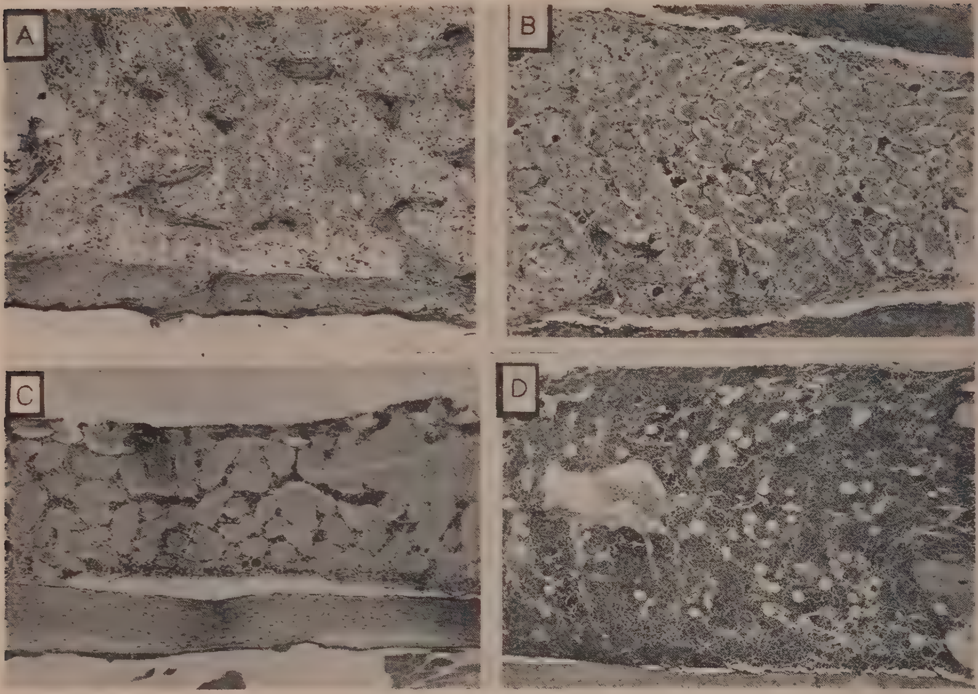

FIG. 2.

Photomicrographs of femoral bone marrow of mice at 4 and 8 days after 1025 r whole body X radiation. HEA × 70. (a) Whole body X radiation without lead protection of the spleen (4-day interval). (b) Whole body radiation without lead protection of the spleen (8-day interval). (c) Whole body X radiation with lead protection of the spleen (4-day interval). (d) Whole body X radiation with lead protection of the spleen (8-day interval). Note that the marrow in A and B is devoid of free hematopoietic cells and consists of diffuse hemorrhage and dilated venous sinusoids; whereas, in C active hematopoiesis exists about the dilated venous sinuses and a normal cellularity exists in D.

hematopoietic tissues occurred sooner in rabbits which had lead protection of either the spleen or the appendix during whole body exposure to 800 r X radiation than in rabbits irradiated without lead protection of the spleen or appendix(4). Ectopic blood formation in the lead protected spleen of rats and mice is greatly intensified after irradiation; whereas, in the lead protected spleen of the irradiated rabbit, ectopic blood formation is minimal and absent in the lead protected appendix of the irradiated rabbits.

*Discussion.* Several possible explanations for these findings may be mentioned. After exposure to whole body X radiation, the irradiated spleen may, by some humoral mechanism, suppress regeneration of hematopoietic tissue in the marrow and lymphatic organs. Perhaps irradiation injury is so great in the group without lead protection of the spleen that failure of hematopoietic recovery is simply a "toxic" phenomenon. Against this latter suggestion is the fact that the weight loss of both irradiated groups closely paralleled each other during the period in which marrow and lymphatic tissue of the spleen-protected group had already begun to regenerate; whereas, no regeneration was apparent in the other. The essentially normal spleen of the lead protected group which quickly assumes a major role in blood formation may reduce radiation toxicity sufficiently

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4. Jacobson, L. O., Marks, E. K., Robson, M. J., and Bethard, W. F., 1949, unpublished data.

to allow for normal hematopoietic regeneration. Since lead protection of either the spleen or the appendix of the rabbit during irradiation allows a more rapid regeneration of the irradiated hematopoietic tissue than in rabbits without such protection, it is obvious that neither is it the spleen specifically nor the ability of the lead protected tissue to produce ectopic blood formation that accounts for the observed phenomenon. These findings strongly suggest that lymphatic tissue exerts a humoral control over hematopoiesis. It might be postulated that the bone marrow which is not normally lymphopoietic, and the thymus would produce a comparable effect were it possible to test this postulate by exclusive protection of these tissues during irradiation. Such experiments are probably necessary before one can assume that the entire hematopoietic system functions as a unit in the maintenance of a steady state. Colonization or seeding from the lead protected lymphatic tissue may actually provide cells from which the irradiated marrow and lymphatic tissue become repopulated. If this latter suggestion were true, then it would follow that the lymphocytes coming from a protected lymphatic organ such as the appendix and going to the bone marrow were capable of giving rise to all types of hematopoietic cells.

If the irradiated lymphatic tissue is acting to temporarily suppress hematopoietic regeneration and recovery under the conditions of these experiments, then the process may be related to the "indirect" therapeutic effect observed in myelogenous leukemia when the spleen alone is irradiated.

It should perhaps be pointed out that lead protection of lymphatic tissue of irradiated animals may possibly reduce "radiation toxicity" in a non-specific manner and that a more rapid regeneration of tissue in the body other than hematopoietic tissue such as skin, gonads, and intestinal mucosa will be apparent on examination.

*Summary and conclusions.* Young adult female mice were exposed to 1025 r whole-body X radiation. The spleens of these mice were mobilized surgically through an abdominal incision and lead protected during irradiation in one group and mobilized but not lead protected during irradiation in another group.

The bone marrow and lymphatic tissues were destroyed and no regeneration was as yet apparent on the tenth day in the animals irradiated without lead protection of the spleen. In the animals with lead protection of the spleen during irradiation, these tissues were never depleted of free hematopoietic cells and the bone marrow and the lymph nodes were normal in cellularity by the eighth day after irradiation.

Several possible interpretations of the role of the spleen under the conditions of these experiments are discussed.

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# Effects of Vitamin $B_{12}$ and Liver Residue on Growth of Hyperthyroid Male Rats.* (17712)

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Available data indicate that requirements for a number of nutrients are markedly increased in the hyperthyroid animal(1). This is particularly true for some of the B vitamins. An increased requirement for thiamine(2,3), pyridoxine(4), pantothenic acid

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* Communication No. 249 from the Department of Biochemistry and Nutrition, University of Southern California.

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4. Drill, V. A., and Overman, R., *Am. J. Physiol.*, 1942, v135, 474.

(4), folic acid (5) and, more recently, vitamin $B_{12}$(6-8) has been demonstrated following the administration of large doses of thyroactive substances. In addition to the above, requirements are increased for at least one additional factor as well. This substance which has been termed the "antithyrotoxic factor of liver" is present in considerable concentration in the water-insoluble fraction of liver (liver residue) and is apparently distinct from any of the known nutrients(9-11). When immature female rats were fed purified rations containing casein as the dietary protein, a marked retardation in growth and inhibition of ovarian development was observed following the administration of massive doses of desiccated thyroid. These effects were completely counteracted by the administration of liver residue but not by supplements of any of the known nutrients including vitamin $B_{12}$ (9,11,12). Betheil and Lardy, however, found that male rats on a similar ration supplemented with thyroid gained significantly more weight following vitamin $B_{12}$ administration than they did if vitamin $B_{12}$ were omitted(8). Since differences in sex may have been responsible at least in part for the diverse growth response, the present experiment was undertaken to determine the comparative effects of vitamin $B_{12}$ and liver residue on the growth and gonadal weight of male rats fed massive doses of thyroid in conjunction with a purified casein-containing ration.

*Procedure and Results.* The basal ration employed in the present experiment consisted of sucrose, 73.0%; casein,[†] 22.0%; salt mixture,[‡] 4.5%; and U.S.P. desiccated thyroid,[§] 0.5%. To each kg of the above were added the following synthetic vitamins: thiamine hydrochloride, 72 mg; riboflavin, 9 mg; pyridoxine hydrochloride, 15 mg; calcium pantothenate, 67.2 mg; nicotinic acid, 60 mg; 2-methyl-naphthoquinone, 5 mg; and choline chloride, 1.2 g.[‖] Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol acetate 1.5 mg and a vitamin A-D concentrate containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.[¶] In addition to the basal ration the following diets were also employed, consisting of basal ration plus each of the following supplements: (1) 30 $\mu$ of vitamin $B_{12}$ per kg of diet, and (2) 10% liver residue powder,[**] added in place of an equal amount of sucrose. A control ration was also employed consisting of the basal ration with thyroid omitted and replaced by an equal amount of sucrose. Forty male rats of the Long-Evans strain were selected at 22 to 25 days of age and an average weight of 50.6 g for the present experiment. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets *ad lib* (10 rats per group). Feeding was continued for 28 days. Results are summarized in Table I.

Findings are in agreement with those previously reported for the female rat(11,12). Massive doses of thyroid retarded the gain in

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9. Ershoff, B. H., *Arch. Biochem.*, 1947, v15, 365.

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11. Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 209.

12. Ershoff, B. H., *J. Nutrition*, 1949, v39, 259.

[†] Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[‡] Sure's Salt Mixture No. 1[13].

13. Sure, B., *J. Nutrition*, 1941, v22, 499.

[§] Thyroid Powder, U.S.P., Armour and Co., Chicago, Ill.

[‖] In view of the increased requirements for thiamine, pyridoxine, and pantothenic acid in the hyperthyroid rat(4), the B vitamins in the present experiment were administered in excessive amounts in order to assure an adequacy of these factors in the diet.

[¶] Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vit. A and 80,000 U.S.P. units of vit. D per g.

[**] Extracted Liver Residue, Wilson Laboratories, Chicago, Ill. This liver fraction consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.

TABLE I.
Effects of Vitamin $B_{12}$ and Liver Residue on the Body and Gonadal Weight of Immature Male Rats fed Massive Doses of Desiccated Thyroid, 10 Rats in Each Experiment.

| Supplements fed with basal ration | % thyroid | Initial body wt, g | Avg gain in body wt after 28 days feeding, g* | Avg wt of testicles, mg* |
|---|---|---|---|---|
| None | .5 | 52.3 | 62.1 ± 5.4<br>(9) | 1.22 ± .12<br>(9) |
| Vit. $B_{12}$ | .5 | 50.6 | 67.6 ± 3.1<br>(9) | 1.46 ± .10<br>(9) |
| Liver residue | .5 | 50.1 | 133.9 ± 4.3<br>(10) | 2.19 ± .09<br>(10) |
| None | .0 | 49.5 | 116.3 ± 6.1<br>(10) | 2.07 ± .07<br>(10) |

The values in parentheses indicate the number of animals which survived and on which averages are based.

* Including standard error of the mean calculated as follows $\sqrt{\frac{\epsilon d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

body and gonadal weight of immature male rats. These effects were completly counteracted by the administration of liver residue at a level of 10% of the diet. Crystalline vitamin $B_{12}$ at a level of 30 $\mu$ per kg of diet was without significant effect. It is apparent that the growth retardation and inhibition of testicular development of male rats fed massive doses of thyroid was due, under conditions of the present experiment, to a deficiency of some factor other than vitamin $B_{12}$. As in the case of female rats, water-insoluble liver residue was a potent source of the missing factor.

In view of the increased nutritional requirements of the hyperthyroid animal, it is recognized that unless precautions are taken to the contrary a multiple dietary deficiency may readily develop as a result of thyroid feeding. Under such circumstances growth may be stimulated by the administration of any one of the missing nutrients, but gain in body weight will not reach normal levels until all the essential nutrients are present in adequate amounts. Betheil and Lardy(8) found that vitamin $B_{12}$ partially counteracted the growth retardation of immature male rats fed massive doses of thyroid in a ration similar to that employed in the present experiment. Gain in body weight was significantly less, however, than that occurring in animals fed a similar diet supplemented with 10% whole liver. It would appear, therefore, that Betheil and Lardy were dealing with a multiple nutritional deficiency. Vitamin $B_{12}$ was one of the deficient factors, but optimum growth was not obtained unless animals were supplied with at least one additional nutrient, present in whole liver powder and presumably identical with the "anti-thyrotoxic factor of liver". Since significant amounts of vitamin $B_{12}$ can be stored in the tissues of the young rat during the period of pregnancy and lactation(14), the occurrence of a vitamin $B_{12}$ deficiency and its extent following thyroid feeding is dependent in considerable degree on the vitamin $B_{12}$ content of the pretest dietary regime. It would appear that differences in the pretest dietary regime or differences in strain requirements for vitamin $B_{12}$ were responsible, at least in part, for the diverse results obtained by Betheil and Lardy from those reported above.

*Summary.* Immature male rats were fed massive doses of thyroid in conjunction with a purified ration containing casein as the dietary protein. A marked retardation occurred both in body and gonadal weight. These effects were completly counteracted by the administration of liver residue. Crystalline vitamin $B_{12}$, however, was without significant effect.

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14. Emerson, G. A., Wurtz, E., and Zanetti, M. E., *Fed. Proc.*, 1949, v8, 381.

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# Electrolyte, Water and Glycogen in Rat Muscle Following Ligation of Major Arteries and Veins.* (17713)

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In order to elucidate the nature of the physiological changes preceding irreversible damage produced by ischemia we have investigated the electrolyte and glycogen content of muscle following frostbite(1), at various periods after the removal of limb tourniquets(2,3) and after vascular occlusion produced by the intra-arterial injection of glass microspheres(4,5). For comparison with these results we have also determined the electrolyte, glycogen and water content of rat muscle following ligation of the major vessels of the hind leg. The latter method does not produce marked alteration in electrolyte or water content of the muscle. Neither gangrene nor permanent functional changes have been observed in the rat. The chemical changes following severe ischemia are dependent upon the duration of the ischemia(2,3). Failure of their appearance after vessel ligation furnishes evidence that in the rat adequate collateral circulation is established in about 3½ hours.

*Methods.* Adult albino rats of the Slonaker-Wistar strain, weighing 150 to 300 g were anesthetized with pentobarbital sodium. The desired vessels were then ligated using the aseptic precautions usually observed in rat surgery(6). After 3 or 4 days the animals were again anesthetized with pentobarbital, 3 to 5 ml of blood were withdrawn from the abdominal aorta, and the anterior tibial and gastrocnemius muscles of both legs were removed for chemical determinations. The muscle was dried to constant weight at 110°C. The dried muscles from the same leg were sometimes combined before ashing. Sodium and potassium were determined by means of the flame photometer (Perkin-Elmer Co., Model 18) on solutions of muscle ash from which the phosphate had been removed(7). Glycogen was determined by the method of Good, Kramer and Somogyi(8) with the modifications suggested by Deane *et al.*(9). Skin temperature of the feet was measured by means of a thermocouple as previously described(10).

*Results.* 1. *Ligation of the femoral artery.* In 5 animals the left femoral artery was ligated a few mm proximal to the superficial circumflex iliac artery 4 days before the animals were killed. The results of the chemical analyses on 4 animals are presented in Table I. A latex mass was injected into the abdominal aorta of the fifth animal. The data show that the mean water, sodium and potassium concentrations are approximately the same in both legs. The glycogen content of the gastrocnemius was greatly reduced in 2 animals, but only slightly reduced in the other 2. The foot temperatures were not significantly different in the 2 legs. All 5 animals had normal use of the leg in which the femoral artery had been ligated. In one animal sodium fluorescein was injected into the tail vein. Observation of its distribution

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* Supported by grants from the John and Mary R. Markle Foundation and the Fluid Research Fund of the Stanford University School of Medicine.

1. Crismon, J. M., and Fuhrman, F. A., *Science*, 1946, v104, 408.

2. Fuhrman, F. A., and Crismon, J. M., *Fed. Proc.*, 1947, v6, 108.

3. Fuhrman, F. A., and Crismon, J. M., in preparation.

4. Crismon, J. M., and Fuhrman, F. A., *Fed. Proc.*, 1947, v6, 93.

5. Crismon, J. M., Watson, J., and Fuhrman, F. A., in preparation.

6. Ingle, D. J., and Griffith, J. Q., Surgery of the Rat, in *The Rat in Laboratory Investigation*, Lippincott, Philadelphia, 1949, 434.

7. Crismon, J. M., *Fed. Proc.*, 1948, v7, 24.

8. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, v100, 485.

9. Deane, H. W., Nesbett, F. B., and Hastings, A. B., Proc. Soc. Exp. Biol. and Med., 1946, v63, 401.

10. Fuhrman, F. A., and Crismon, J. M., *J. Clin. Invest.*, 1947, v26, 236.

TABLE I.
Skin Temperature and Chemical Analyses of Muscle Following Ligation of the Major Arteries and Veins of the Hind Leg of the Rat.
I. Left femoral artery ligated.
II. Left common iliac artery and vein ligated.
III. Left femoral, saphenous, and popliteal arteries and veins ligated.
All constituents are per 100 g dry tissue.

| | I | | II | | III | |
|---|---|---|---|---|---|---|
| | Right | Left | Right | Left | Right | Left |
| Water (g): | | | | | | |
| Gastrocnemius | 306.3 | 308.3 | 303.8 | 306.1 | 305.4 | 326.1 |
| Anterior tibial | 299.2 | 299.5 | 294.2 | 296.8 | 295.3 | 346.4 |
| Sodium (mEq): | | | | | | |
| Gastrocnemius | 3.8* | 4.1* | 8.6 | 9.3 | 8.8 | 11.7 |
| Anterior tibial | 4.5* | 4.9* | | | | |
| Potassium (mEq): | | | | | | |
| Gastrocnemius | 25.7* | 23.7* | 37.7 | 37.2 | 42.2 | 43.8 |
| Anterior tibial | 25.1* | 25.5* | | | | |
| Glycogen (g): | | | | | | |
| Gastrocnemius | 1.69 | 1.25 | 1.14 | 1.72 | | |
| No. of animals | 4 | | 3 | | 2 | |
| Skin Temp. °C | | | | | | |
| Foot: dorsal | 30.6 | 30.1 | 30.1 | 29.7 | | |
| plantar | 31.2 | 30.4 | 31.0 | 30.3 | | |
| No. of animals | 4 | | 6 | | | |

* Determined without phosphate precipitation(7) and therefore low.

under ultraviolet illumination showed that it appeared at the same time in both legs and was of about the same brilliance.

2. *Ligation of the common iliac artery and vein.* In 6 animals the common iliac artery and its accompanying vein were ligated proximal to the hypogastric artery. Several of these animals showed a temporary partial loss of function of the left leg on the first day after ligation, but on the fourth day after ligation all animals had normal use of the leg. The fluorescein test was performed in 3 animals, at 1, 2 and 4 days after ligation. During the first 2 minutes after injection, the left leg was slightly less fluorescent than the right in all 3 animals. Reduced skin temperatures were found in the left feet of 2 animals on the first day after ligation. In one animal the left foot was 3°C, in the other animal 7°C cooler than the right foot. No differences in skin temperature between the 2 feet were observed on the second or subsequent days after ligation. A similar transient fall in skin temperature has been reported in the dog following ligation of the external iliac artery(11) or the femoral artery proximal to the deep femoral branch(12). Three animals were killed after 4 days for chemical analyses of the tissues. The results are given in Table I. As in Series I there was little difference in water, sodium or potassium concentrations in the 2 legs. In contrast to the animals in which the femoral artery was ligated, in this series the left gastrocnemius contained slightly more glycogen than did the right. The foot temperatures of the right and left feet in the 6 animals in this series and the 4 in Series I were combined for statistical analysis. The method of paired differences showed that the temperatures of the right and left feet were not significantly different 4 days after ligation, ($P<0.4$ for dorsal aspect and $<0.2$ for the plantar).

3. *Ligation of femoral, popliteal and saphenous arteries and veins.* In 4 animals in which the left femoral, popliteal and saphenous arteries and their accompanying veins were ligated in the popliteal fossa, no functional changes were observed. After 3 days chemical analyses were made on the muscles of 2 animals, and 2 were injected with latex for study of collateral circulation. The in-

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11. Mulvihill, D. A., Harvey, S. C., and Doroszka, V., *Am. J. Surg.*, 1931, v13, 431.

12. Reichert, F. L., PROC. SOC. EXP. BIOL. AND MED., 1932, v29, 473.

crease in sodium and water content of the gastrocnemius indicated in Table I is the result of change in only one of the animals. In this animal the extra sodium in the ligated leg amounted to 6.0 mEq and the extra water to 34.7 g per 100 g dry tissue. Since the plasma sodium concentration was 148.5 mEq per liter, 5.15 mEq of Na, or 86% of that found, could be accounted for on the basis of the presence of edema fluid containing sodium at the same concentration as that of plasma. Since this is probably within the limits of error of the methods used there is no evidence for the accumulation of sodium in excess of water.

*Discussion.* Workers disagree as to the extent of gangrene following ligation of large arteries in the hind leg of the rabbit(13,14,15,16); following ligation of the common iliac artery gangrene has been reported to result in 10%(15) to 71.5%(13) of the animals. In the dog gangrene was not produced by ligation of the external iliac artery, or of the artery and vein(11). To produce it consistently in the dog Murray *et al.*(17) found that it was necessary to ligate not only the external iliac artery but also 12 other arteries supplying the muscles of the hind leg. In the rabbit when gangrene did occur it appeared the second day after ligation(13). In the experiments reported here the rats were only observed for 4 days, but it would seem that gangrene would have been observed within that time if it were to develop. It has been shown previously that a wide variety of severe injuries, produced by frostbite(1), anoxia (18), burning(19) or tourniquets(19,2), result in the accumulation in the injured tissues of sodium in excess of water. This phenomenon appears to be a non-specific event. It is apparent from the table that in the rat extensive sodium accumulation, of the type previously reported to occur after other injuries, did not result from the ischemia produced by ligation of the major arteries of the limb. Since neither gangrene nor permanent functional impairment occurred it is apparent that collateral circulation in this species is sufficient to maintain the integrity of the leg muscles after loss of their major blood supply.

*Summary.* Ligation of the femoral artery (Series I), the common iliac artery and vein (Series II) or the femoral, popliteal and saphenous arteries and veins (Series III) in albino rats resulted in only slight alteration in the sodium, potassium and water content of the gastrocnemius and anterior tibial muscles 4 days after occlusion. Muscle glycogen was reduced in 2 out of 4 animals in Series I. Edema occurred in 1 animal in Series III, but the extra sodium found in the muscle could be accounted for by the increase in extracellular fluid. No gangrene or permanent functional changes were observed.

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13. Brooks, B., and Martin, K. A., *J. A. M. A.*, 1923, v80, 1678.

14. Holman, E., and Edwards, M., *J. A. M. A.*, 1927, v88, 909.

15. Wilson, W. C., *Brit. J. Surg.*, 1933, v20, 393.

16. Holman, E., *Surg., Gynec. and Obstet.*, 1942, v75, 183.

17. Murray, G., Simpson, J. S., and Watters, N. A., *Surgery*, 1946, v20, 315.

18. Darrow, D. C., and Engel, F. L., *Am. J. Physiol.*, 1945, v145, 32.

19. Fox, C. L., and Baer, H., *Am. J. Physiol.*, 1947, v151, 155.

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# Antithromboplastic Activity of Monosodium Alpha Tocopherol Phosphate and Inositol Phosphatide.* (17714)

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Thomas(1) showed that intravenous injections of thromboplastin, a sedimentable tissue component, into mice caused a characteristic response consisting of ataxia, convulsions and coma. The easy reproduction of these symptoms with small, relatively constant quantities of thromboplastin provided a more or less quantitative method for titrating the thromboplastic activity of these suspensions. Thomas further showed that this response could be prevented by both heparin and congo red. This paper deals with the inhibition of thromboplastin by two other substances, monosodium alpha tocopherol phosphate† and inositol phosphatide‡. Overman(2) reported the antithromboplastic action of inositol phosphatide on the basis of *in vitro* work. No similar studies seem to have been carried out with the alpha tocopherol preparation.

*Method.* These experiments were carried out on mice, each weighing from 12 to 14 g. Mixed brain and lung thromboplastin§ was standardized by the method of Thomas(1) as follows: It was diluted with distilled water to make up concentrations varying from 1:10 to 1:50. Each solution was incubated for 60 minutes at 37.5°F and was injected into the tail vein of a mouse, which was observed for the characteristic response of ataxia, convulsions and coma. The minimum effective dose which produced this response was 0.2 ml of thromboplastin in 1:30 dilution. Alpha tocopherol was then added to a 1:20 thromboplastin solution in amounts varying from 7.5 to 100 $\gamma$. Immediate injection of the solution into the tail veins of mice in 0.2 ml aliquots was ineffective in inhibiting the characteristic response to thromboplastin. When, however, the solution was incubated for 60 minutes at 37.5°C and was similarly injected, it was found that 15 $\gamma$ of alpha tocopherol phosphate was effective for this purpose, while 7.5 $\gamma$ per 0.2 ml aliquot lacked sufficient antithromboplastic activity to prevent the reaction.

*Results.* Similar experiments with inositol phosphatide showed (1) that without incubation the test solution had no inhibitive effect and (2) that 36 $\gamma$ of the substance per 2 ml aliquot was capable of inhibiting the response of a mouse to a 1:20 dilution of thromboplastin when the same aliquot was used. At 18 $\gamma$ no antithromboplastic activity was observed. Since it had been shown that both monosodium alpha tocopherol phosphate and inositol phosphatide were antithromboplastic in the concentrations used in this experiment after the solutions had been incubated, another experiment was done to determine whether the antithromboplastic action would occur if the test solutions were injected into the mouse prior to the injection of a 0.2 ml aliquot of the minimum effective dose of thromboplastin. After 15 to 100 $\gamma$ of monosodium alpha tocopherol phosphate per 0.2 ml aliquot had been injected intravenously into the mouse, either 5 or 30 minutes were permitted to elapse before 0.2 ml of the 1:20 thromboplastin dilution was injected into the same mouse. All of the mice showed the characteristic response to thromboplastin, no antithromboplastic activity being demonstrated in any of them. At 1,000 $\gamma$ inhibition of thromboplastin occurred after a 5 and a 30 minute interval.

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* This work has been aided by a grant from the Warren-Teed Products Co., Inc.

1. Thomas, L., *Bull. Johns Hopkins Hosp.*, 1947, v81, 1.

† Supplied by Mr. Rudolph Frundt of the Warren-Teed Products Co., Inc.

‡ Supplied by Dr. George Mast of C.S.C. Pharmaceuticals.

2. Overman, A. S., and Wright, I. S., *J. Biol. Chem.*, 1948, v174.

§ Sol U Plastin supplied by Mr. Blanchard of Schaffiin and Co.

TABLE I.

Effect of Alpha Tocopherol Phosphate and Inositol Phosphatide on the Reaction to Thromboplastin in Mice.

| Thromboplastin dilution | Diluent | | | | | | |
|---|---|---|---|---|---|---|---|
| | Distilled $H_2O$ | a$TPO_4$ 7.5 γ per 0.2 ml | a$TPO_4$ 15 γ per 0.2 ml | a$TPO_4$ 15 γ/0.2 ml Fibrinogen 50 mg/0.2 ml | Inositol phosphatide 18 γ/0.2 ml | Inositol phosphatide 36 γ/0.2 ml | Inositol phosphatide 36 γ/0.2 ml Fibrinogen 50 mg/0.2 ml |
| 1/40 | 0 0 0 0 0 0 | | | | | | |
| 1/30 | + + + + + + | | | | | | |
| 1/20 | + + + + + + | + + + | 0 0 0 | + + + | + + + | 0 0 0 | + + + |

* 0, no reaction. +, positive reaction. Symbols indicate number of mice used in each test.

Similar studies were carried out with inositol phosphatide in 36 γ amounts per 0.2 ml aliquot. No antithromboplastic activity could be observed. When, however, the same experiment was repeated with injections of 72 γ, antithromboplastic activity was observed at both the 5- and the 30-minute interval following the injection of the inositol phosphatide. The clotting time of the blood, which had not been affected when monosodium alpha tocopherol phosphate was used, was raised to 9 minutes or higher in the mice tested. Finally since fibrinogen‖ is known to block the antithrombic action of monosodium alpha tocopherol phosphate, its effect on the antithromboplastic activity of this substance and of inositol phosphatide was tested.

The control group of mice used in this experiment were tested with injections of 100 γ of alpha tocopherol per 0.2 ml incubated with thromboplastin in 1:20 dilution. None of the animals showed the typical reaction to thromboplastin. When, however, the same solution was tested in mice with the addition of 50 mg of fibrinogen per 0.2 ml aliquot, the typical reaction did occur. The same experiment was carried out with inositol phosphatide in concentration of 36 and 72 γ, respectively. With each concentration the addition of fibrinogen blocked the protective effect of the inositol phosphatide solution. The end points in these experiments were checked, using at least 3 mice for each test. A 100% effect was noted with each concentration of solution, as recommended by Thomas (Table I).

*Discussion.* Since it has been suggested that the activity of thromboplastin may be the result of its effect on surface tension, it was postulated that the antithromboplastic effect of the substances tested in these experiments might result from their ability to reduce surface tension. Studies done by Mr. Rudolph Frundt of Warren-Teed Products Co. showed a value of 33 dynes $cm^2$ for a 10 mg/ml solution of monosodium salt of alpha tocopherol phosphate and a value of 45.4 dynes $cm^2$ for a 3.571 mg/cc solution of inositol phosphatide, compared with a value of 72.8 dynes $cm^2$ for distilled water.

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‖ Fraction I from bovine plasma, purchased from Armour & Co., was used.

These observations suggest the possibility (1) that thromboplastin presents a surface which can adsorb monosodium alpha tocopherol phosphate and inositol phosphatide, (2) that the utilization of this surface by these surface-acting agents explains why their injection into mice in a solution of thromboplastin prevents the typical reaction which normally occurs in these animals following the injection of thromboplastin alone, and (3) that the addition of fibrinogen to the solution interferes with this protective action because of the surface presented by fibrinogen molecules.

*Summary.* Experimental studies on mice showed that the typical reaction of these animals to the injection of thromboplastin can be prevented if monosodium alpha tocopherol phosphate or inositol phosphatide is incubated with the thromboplastin before it is injected. When fibrinogen is added to the test solution, the inhibitory effect of each of these substances is lost. It is possible that alterations in surface tension may explain these phenomena.

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## Use of Maxted's Method for Group Classification of Hemolytic Streptococci.*† (17715)

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Maxted(1) has recently described a method for grouping beta-hemolytic streptococci in which the streptococci are lysed by a substance, presumably an enzyme, produced by a strain of *Streptomyces albus*. Such lysates contain the streptococcal C substance and can be used as antigens in the precipitin reaction for group classification. The simplicity, reliability and usefulness of Maxted's technique have been confirmed in a study of 1,010 consecutive strains of streptococci.

*Methods.* The strain of *Streptomyces albus*, obtained from Maxted, was grown on the following medium which is a modification of that described previously(1).

| | |
|---|---|
| Bacto-peptone (Difco) | 0.5 g |
| Yeast extract, desiccated | 0.6 ” |
| Dibasic sodium phosphate, hydrous | 0.2 ” |
| Glucose | 0.2 ” |
| Fildes' extract(2) | 2.5 ml |
| Agar | 1.25 g |
| Distilled water | 100.00 ml |

These constituents, with the exception of the Fildes' extract, were mixed and dispensed into a 1000 ml Roux bottle. After sterilization at 120°C for 20 minutes, the bottle was cooled to about 40°C, the Fildes' extract added, and the agar medium allowed to gel with the bottle in the horizontal position. The surface was inoculated evenly with 1 ml of a heavy suspension of *Streptomyces albus* spores in 0.85% sodium chloride solution. After incubation at 35°C for 5 days, the culture was frozen at –10°C for 12 hours and then allowed to thaw at room temperature. This procedure separated the agar gel of the

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† The authors take pleasure in acknowledging the technical assistance of Mary E. Riner and Hallie E. Wannamaker. The authors wish to express their appreciation to Dr. Armine Wilson for his advice on methods of streptococcal classification.

1. Maxted, W. R., *Lancet*, 1948, v255, 255.

2. Fildes, P., *Brit. J. Exp. Path.*, 1920, v1, 129.

medium from the fluid containing the active substance. The fluid was strained through gauze and the pH adjusted to 7.5 with 1 N hydrochloric acid. The extract was then filtered through a Seitz pad and phenol was added to a concentration of 0.5%. Preparations retained their activity for several months at 4°C. The potency of an extract was determined by its ability to lyse hemolytic streptococci. A small loopful of an 18 hour growth from a blood agar plate was inoculated into 0.25 ml of the preparation and the mixture placed in a water bath at 50°C. Lysis occurred in less than 30 minutes with very potent extracts, whereas weak preparations required as long as 24 hours.

The clear lysate, prepared with an active extract, was then used as antigen in a precipitin reaction with group specific rabbit antisera by the capillary tube technique(3). In some instances where the original suspension was very heavy and complete clearing had not occurred after long incubation (24 hours), it was possible to centrifuge the suspension and use the clear supernatant fluid as antigen. The precipitin reaction in the capillary tubes was graded according to standards previously established(3). Readings were made at the end of 1 hour of incubation at 37°C and again after overnight refrigeration at 4°C.

The 1010 consecutive strains of streptococci herein reported were isolated from the throats of normal subjects and of patients with streptococcal infections. Colonies exhibiting the *beta* type of hemolysis on medium containing 5% horse blood were transferred to medium containing 5% sheep blood. All those showing *beta* hemolysis (either on the surface or around a stab into the agar) on sheep blood medium were included in this study(4).

*Results.* Of the 1010 consecutive strains of hemolytic streptococci, 998 were successfully classified by Maxted's method as follows: 762 were group A, 49 group B, 94 group C, 3 group D, 4 group F, and 86 group G. There were 12 strains which could not be identified with available antisera. Nine of these 12 strains were tested with antisera for groups A, B, C, D and G. The other 3 strains were tested in addition with antisera for groups E, F, H and L. Attempts were made to confirm the results of Maxted's method by either the formamide technique(5), the hot acid method(3), or both. When the 12 strains which failed to group by Maxted's technique were examined by one or both of these methods, 11 failed to give a precipitin reaction. One strain was found to be group G by the hot acid method. This strain was not tested by the formamide method or reexamined by Maxted's technique.

Of the 998 strains which were successfully classified by Maxted's method, 791 were examined by one or both of the older methods (Table I). The first 294 strains were examined by the formamide method and 36 strains were classified by both the formamide and hot acid methods. No disagreements were obtained. An additional 461 strains were examined by the hot acid method. Of these 461 strains, 415 were successfully classified using the hot acid method and among these there were no discrepancies with the findings by Maxted's technic. Forty-six strains failed to group by the hot acid method in one attempt. These strains were lost before the tests could be repeated.

*Discussion.* The reliability of Maxted's method was determined by performing parallel tests on 803 strains by one of the two techniques already in general use. Whenever positive precipitin reactions were obtained by both of the methods being compared, there was complete agreement in classification. The 47 discrepancies were instances in which a positive result was obtained by one method and a negative by the other. None of these discrepancies occurred with the formamide method; all occurred when comparison was being made with the hot acid method. Experience in this laboratory with the hot acid extracts for group classification was not al-

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3. Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, v78, 127.

4. Feller, A. E., Badger, G. F., Dingle, J. H., Hodges, R. G., and Rammelkamp, C. H., *J. Clin. Invest.*, 1949, v28, 780.

5. Fuller, A. T., *Brit. J. Exp. Path.*, 1938, v19, 130.

TABLE I.
Comparative Findings by Other Grouping Technics on 791 Strains Successfully Grouped by the Maxted Technic.

| Classification by the enzyme method Group | Method or methods used for comparison | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Formamide and hot acid | | | Formamide | | | Hot acid | | |
| | No. strains | Same group | Neg. reaction | No. strains | Same group | Neg. reaction | No. strains | Same group | Neg. reaction |
| A | 35 | 35 | 0 | 287 | 287 | 0 | 249 | 249* | 0 |
| B | 0 | 0 | 0 | 0 | 0 | 0 | 45 | 35 | 10† |
| C | 1 | 1 | 0 | 3 | 3 | 0 | 82 | 69 | 13† |
| D | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 3† |
| F | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 0 |
| G | 0 | 0 | 0 | 4 | 4 | 0 | 79 | 59 | 20† |
| Total | 36 | 36 | 0 | 294 | 294 | 0 | 461 | 415 | 46† |

* In approximately 18% of these group A strains it was necessary to repeat the test before a precipitin reaction was obtained.

† These non-group A strains were lost before a second hot acid extract could be prepared.

together satisfactory. In many instances the precipitin reaction obtained was minimal and transient. A stronger reaction could be obtained by diluting the extract, by using different lots of antisera or by preparing a new extract. All strains which were so checked eventually gave a positive result with the hot acid extract which confirmed that obtained with the Maxted extract. Using the hot acid technic, it was necessary to repeat the tests on approximately 18% of the strains of streptococci as compared to approximately 2% with the formamide technique and less than 1% with Maxted's technique.

Stronger precipitin reactions are usually obtained with the Maxted extracts than with the formamide and hot acid extracts, thus simplifying the reading of the tests. The reactions employing the Maxted extracts should be read after one hour of incubation since cross reactions may develop after overnight refrigeration.

Of practical importance is the fact that group classification by Maxted's technique can be completed within a few hours after the streptococci have been isolated in pure culture on a blood agar plate. Also, the only procedure required for making the extracts is a transfer of the organisms into the *Streptomyces* preparation.

*Summary.* Experience with 1010 consecutive strains of hemolytic streptococci examined by Maxted's grouping technique is presented. Classification by this method was successful with 998 strains. Comparison with the older formamide and hot acid techniques suggests that Maxted's method is reliable and is simpler to perform.

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## Use of Mucin in Experimental Infections of Mice with *Histoplasma capsulatum*. (17716)

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The use of mucin as a resistance-lowering substance for bacterial and virus infections recently has been reviewed in detail by Olitzki (1). No published data have been cited, however, to suggest that mucin has ever been employed as an adjuvant in establishing experi-

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1. Olitzki, L., *Bact. Rev.*, 1948, v12, 149.

TABLE I.
Effect of Mucin on Virulence of *H. capsulatum* for Mice.

| Strain No. | Group | Challenge dose of *H. capsulatum* Million | No. days after inj. | No. deaths in mice inoculated with organisms suspended in: | |
|---|---|---|---|---|---|
| | | | | Saline | 5% mucin |
| G-2 | A | 35 | 9 | 0/10[*] | 10/10 |
| | B | 7 | 20 | 0/10 | 10/10 |
| | C | 0.7 | 30 | 0/10 | 3/10 |
| G-5 | A | 35 | 30 | 0/10 | 8/10 |
| | B | 7 | 30 | 0/10 | 4/10 |
| | C | 0.7 | 30 | 0/10 | 1/10 |
| G-6 | A | 35 | 30 | 0/10 | 5/10 |
| | B | 7 | 30 | 0/10 | 1/10 |
| | C | 0.7 | 30 | 0/10 | 0/10 |
| G-8 | A | 35 | 11 | 0/10 | 10/10 |
| | B | 7 | 17 | 0/10 | 10/10 |
| | C | 0.7 | 30 | 0/10 | 4/10 |
| G-8 control | † | 7 | 30 | — | 0/20 |
| Mucin control | ‡ | — | 30 | — | 0/20 |

[*] In this and other tables the numerator represents the number of deaths and the denominator the total number of mice in the experimental groups.

† Organisms were autoclaved before suspending in mucin.

‡ 1 ml of 5% mucin was administered by the intraperitoneal route.

mental infections with the pathogenic fungi. The evaluation of certain chemotherapeutic agents in *Histoplasma capsulatum* infections has been restricted by the relative resistance of white mice to this organism administered by the intraperitoneal route. In order to study the action of chemotherapeutic agents in the treatment of experimental histoplasmosis, it was therefore first necessary to devise an infective technic that would insure consistently high rates of infection and mortality in untreated mice. The successful employment of mucin as an enhancing agent in certain bacterial and virus infections suggested the desirability of investigating its capacity to "enhance" the virulence of *H. capsulatum* for mice.

*Methods.* Inocula were prepared from yeast phase cultures of *H. capsulatum* grown on glucose cystine blood agar(2) for 4 days at 37°C. The growth was washed from the slants and diluted with sterile normal salt solution to yield a density of 0.500 (±.010) at 650 $\gamma$ on the Coleman Junior Spectrophotometer. This suspension, from which all subsequent dilutions were prepared contained approximately 70 million organisms per ml by direct haemocytometer count. Cultural characteristics of the organism in this growth phase made it impracticable to attempt to determine the actual number of viable cells by plate count. The 5% gastric mucin employed was prepared by the Department of Biologic Products of this institution according to the method they routinely use, which is as follows: Granular hog mucin (Wilson, Type 1701-W) is suspended to 5% concentration in distilled water with the aid of a Waring blendor. The suspended material is kept in the refrigerator for 36 hours with occasional shaking, then warmed and adjusted to pH 7.2, filtered through gauze and cotton, and sterilized by heating for 30 minutes in a boiling water bath 3 times within a period of 24 hours. Tests for sterility, toxicity and "virulence-enhancing" activity (in *Salmonella typhosa* infections of mice) are routinely performed. White Swiss mice (Bagg strain) weighing 14-17 g were used throughout these studies and were distributed in groups of 5 mice each in individual jars by a random

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2. Campbell, Charlotte C., *J. Bact.*, 1947, v54, 263.

TABLE II.
Summary of Results of 5 Experiments. Mortalities in Mice Infected with the G-8 Strain of *H. capsulatum* Suspended in Mucin.*

| No. days after injection | Groups | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | | B | | C | | D | | E | | Total | |
| | No. | %† | No. | % | No. | % | No. | % | No. | % | No. | % |
| 7 | 0/40 | 0 | 0/37 | 0 | 0/39 | 0 | 1/40 | 2.5 | 0/47 | 0 | 1/203 | 0.49 |
| 14 | 22/40 | 55 | 18/37 | 48.6 | 17/39 | 43.6 | 19/40 | 47.5 | 25/47 | 53.3 | 101/203 | 49.7 |
| 21 | 30/40 | 75 | 25/37 | 67.5 | 22/39 | 56.4 | 31/40 | 77.5 | 31/47 | 66 | 139/203 | 68.4 |
| 30 | 34/40 | 85 | 28/37 | 75.7 | 26/39 | 66.6 | 31/40 | 77.5 | 39/47 | 83 | 158/203 | 77.8 |

See Table I for legend.
* Inoculum contained approximately 3.5 million organisms.
† % deaths.

procedure.

*Results.* The results obtained with saline and mucin suspensions of 4 yeast phase strains of *H. capsulatum* (G-2, G-5, G-6 and G-8)* are compared in Table I. Challenge doses were administered to 4 groups of 60 mice each. Ten mice in each group received comparable volumes of the organism suspension in 5% mucin, and 10 mice equal dilutions in saline, representing 35 million, 7 million and 700,000 organisms, respectively. Challenge doses of the G-2 strain of *H. capsulatum* suspended in 5% mucin killed all mice in groups A and B (Table I), while in group C only 3 of 10 mice died during the 30 day period. The mortality pattern obtained with the mucin suspension of strain G-8 was similar to that of G-2, but strains G-5 and G-6 administered in mucin were less effective. While it is evident that the "virulence-enhancement" activity of mucin suspensions varied with different strains of the organism it should be noted that no deaths occurred among animals receiving equivalent challenge doses suspended in saline.

To exclude the possibility that neither the mucin nor the mucin-organism suspension was inherently toxic, 2 control groups of 20 mice each were included (Table I). The mice in one group were each injected with 7.0 million G-8 organisms which had been killed by autoclaving before suspending in mucin. Mice in the second control group were inoculated with 1 ml of the 5% mucin suspension which was to be employed as diluent for inocula in this and subsequent experiments reported in this paper. All mice in both control groups survived the 30-day observation period.

Since human histoplasmosis frequently displays a clinical picture of chronicity it was believed that more effective evaluation of chemotherapeutic agents could be obtained if a challenge dose were established which would consistently produce mortality in excess of 50% only after a 2 to 3 week period following injection. A challenge inoculum that would serve this purpose was derived from additional pilot studies with strain G-8. This inoculum consisted of 0.5 ml of a 1:10 dilution of the organism suspension in mucin (approximately 3.5 million organisms). The results of 5 experiments performed at monthly intervals in which this challenge dose was employed are shown in Table II. A total of 210 mice, divided into 4 groups of 40 mice each and 1 group of 50, were inoculated and observed for periods of 30 days. Occasional deaths occurring within the first 3 days after injection were believed not to have been the result of *H. capsulatum* infection and were not included in the final calculations. Mortalities in the various groups varied from 0 to 2.5% by the 7th day, from 43.6 to 55% by the 14th day, from 56.4 to 77.5% by the 21st day and from 66.6 to 85.0% by the 30th day. The average death rates were 0.49, 49.7, 68.4 and 77.8% at 7, 14, 21 and 30 days, respectively.

The marked difference between mucin and saline suspensions observed in the early ex-

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* The G-2, G-5 and G-6 strains of *H. capsulatum* are from the collection of Dr. N. F. Conant, Duke University. The G-8 strain, a human isolate, was identified at this institution.

TABLE III.
Comparison of Mortalities in Mice Receiving Equivalent Challenge Doses of *H. capsulatum* (G-8) Suspended in Mucin and Saline.*

| No. days after injection | Groups |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | F |  |  |  | G |  |  |  | H |  |  |  | Total |  |  |  |
|  | Saline |  | Mucin |  | Saline |  | Mucin |  | Saline |  | Mucin |  | Saline |  | Mucin |  |
|  | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| 14 | 0/40 | 0 | 19/40 | 47.5 | 1/47 | 2.1 | 25/47 | 53.3 | 0/20 | 0 | 10/20 | 50. | 1/107 | 0.9 | 54/107 | 50.5 |
| 21 | 1/40 | 2.5 | 31/40 | 77.5 | 2/47 | 4.3 | 31/47 | 66 | 0/20 | 0 | 13/20 | 65 | 3/107 | 2.8 | 75/107 | 70.1 |
| 30 | 1/40 | 2.5 | 31/40 | 77.5 | 2/47 | 4.3 | 39/47 | 83 | 0/20 | 0 | 15/20 | 75 | 3/107 | 2.8 | 85/107 | 79.4 |

See Table I for legend.
* Inoculum consisted of approximately 3.5 million organisms.

periments was further substantiated with 3 additional groups of mice. These groups consisting of 40, 50 and 20 mice, respectively, were inoculated with 0.5 ml of saline suspension containing approximately 3.5 million organisms. Inoculations for groups F and G (Table III) were performed simultaneously with groups C and D (Table II). The resultant mortalities from mucin and saline suspension inocula are compared in Table III. Administration of *H. capsulatum* in mucin resulted in the markedly superior mortality rate of 79% as compared to that of 2.8% observed in animals receiving the organisms in saline.

Throughout the studies reported in this paper necropsies were performed on animals succumbing to the infection as well as those surviving the 30 day period. *H. capsulatum* was consistently recovered from the spleens of mice inoculated with mucin suspensions of the organism but was rarely isolated from those receiving the organisms suspended in saline.

*Summary and conclusions.* *H. capsulatum* in the yeast phase when suspended in 5% hog gastric mucin and administered intraperitoneally into white mice caused a high percentage of fatal infections. Equivalent challenge doses of the organism suspended in saline rarely caused death. It was observed that by varying the number of organisms administered in mucin the mortality rate as well as the time interval between injection and death could be altered to suit the experimental requirements.

Certain investigations on experimental histoplasmosis heretofore have been limited by the relative resistance of white mice to infection with *H. capsulatum.* "Virulence-enhancement" by mucin offers a means of extending the scope of such studies.

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# Radioiron Excretion by the Skin and Kidney of Dogs. (17717)

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Many publications support the concept that the body absorbs iron in significant amounts as a rule only when its iron stores are depleted(1-3), and that small amounts of iron escape in the urine and bile(4). A recent article(5) states that human subjects under "minimal sweating" conditions can excrete 6.5 mg of iron per day, or 13% of the iron ingested. Since the dog has been used in many experimental studies of iron metabolism it seemed pertinent to measure the iron excretion by the skin in this species which is nearly devoid of sweat glands.

The experiments tabulated below indicate clearly that no significant amount of radioactive iron is excreted by the dog's skin when iron is given by mouth or vein.

*Methods.* The radioiron procedures and other methods used in this laboratory have been described elsewhere(6). The isotopes used consisted almost entirely of $Fe^{55}$. The dogs were thoroughly scrubbed in a baby bathinette, using tap water and a commercial detergent. The entire washings were collected, evaporated to a small volume, and transferred to Kjeldahl flasks for wet ashing with sulfuric and perchloric acids. Prior to electroplating, the iron was extracted with isopropyl ether(7). Urine and feces were similarly treated. Intravenous iron solutions were prepared in gelatin(8). The dogs used were all normal female mongrels with normal hematocrits and presumably adequate iron stores. Dog 43-141 had a year previously received radioiron and had absorbed and utilized a small amount. The other dogs had received none. During the course of the experiments, the animals were caged in the laboratory which was usually around 26°C. No attempt was made to control the humidity.

*Experimental observations.* Routes of administration and radioiron recovery data appear in Table I. The findings are in harmony with work published elsewhere. In the first 2 experiments in Table I, only 0.03 and 0.02% of radioactive iron could be found in the washings taken one day after the intravenous injection of 5.23 and 5.03 mg of iron containing the isotope. In the second experiment only 0.01% could be found after a week.

It will be noted that the washings taken prior to the iron administration in the first experiment contained 900 counts per minute of radioiron. This was thought at the time to have been derived from the radioiron administered at an earlier date. To our surprise, however, the washings taken prior to iron administration in Exp. 2 contained a similar amount of radio-activity. A check revealed that the separatory funnels used in the ether extraction were slightly contaminated with radioiron. Consequently, the traces of radioiron found in the washings of these 2 experiments may have come partially or entirely from this source. Since even traces of iron contaminating the glassware are picked up—710-418 counts per minute or 0.0016-0.0009 mg of radioiron in Exp. 1 and 2—it is scarcely probable that any significant amount of iron eliminated by the skin could escape detection.

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TABLE I.

Recovery of Radioiron in Body Washings and Urine Following Intravenous and Oral Administration of Radioiron.

| Exp. | Dog | Wt in kilo | Hematocrit, % | Route of iron administration | Radioiron, mg* | Body washings | | | | | Urine | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Before iron | After iron administration | | | | | |
| | | | | | | | One day | | One week | | | |
| | | | | | | Counts per min. | Counts per min. | % of dose | Counts per min. | % of dose | Counts per min. | % of dose |
| 1 | 43-121 | 12 | 49 | i.v. | 5.23 | 900 | 710 | .03 | — | — | 37,000 | 1.6† |
| 2 | 49-61 | 12 | 51 | i.v. | 5.04 | 540 | 418 | .02 | 229 | .01 | 35,200 | 1.6† |
| 3 | 49-131 | 11.7 | 54 | oral | 10.6 | 0 | 1240 | .02 | 720 | .02 | 3,000 | 0.06‡ |

58% of the dose in Exp. 3 was found in the feces.

* The activity of the iron used was about 450,000 counts per min. per mg.

† Urine collected for 24 hrs.

‡ ,, ,, ,, 48 ,,

In Exp. 2 determination of total plasma iron 5 minutes after the injection gave a value of 554 γ per 100 ml of which 442 γ per 100 ml could be accounted for from the administered radioiron. Since only traces of radioiron might have been excreted through the skin following intravenous administration, oral administration was tested. A third dog weighing 11.7 kilos was given 10.6 mg of radioiron as ferric chloride by stomach tube (Exp. 3). New separatory funnels were used and no activity was found in the washings prior to radioiron feeding. Only 0.02% of the dose was found in the washings taken at one day and one week. The plasma radioiron one hour after feeding was 15 γ per 100 ml. In one week 2.5% of the dose was found in the circulating red cells.

In Exp. 3, 58% of the dose was found in the feces collected during a 3-day period. This low value(1) probably resulted from too short a collection period. Since no precautions were taken to prevent contamination of the dog by feces, other than by their prompt removal from the cage, some, if not all, of the radioiron found in the washings could have come from this source. Urinary radioiron data are given in the last column of Table I. In Exp. 1 the urine was collected by catheterization; in Exp. 2 and 3 it was collected in a metabolism cage. In each of the 2 tests following intravenous injection the 24-hour urines contained 1.6% of the radioiron administered. After oral feeding, only 0.06% of the administered radioiron appeared in the urine in 48 hours. The high values following intravenous administration are presumably due to the higher plasma iron values for the few hours following injection.

The urinary excretion of radioiron was many times that found in the dermal washings, even assuming that all such radioiron was excreted by the skin.

*Discussion.* From the data presented it is quite obvious that an insignificant amount of radioiron, if any, appears in washings from dogs given a single dose of radioiron by vein or mouth. The maximum amount found corresponded to 0.03% of the dose and may well have resulted largely from contamination of the glassware in the first 2 experiments, or of the skin by feces in the third. It would seem reasonable to assume that any dermal loss of iron would be mediated in part at least by the plasma transport mechanism, yet in the intravenous experiments in which the plasma iron was raised to very high levels, no appreciable loss through the skin occurred.

It should be noted that an oral dose of 10 mg of iron to a 10 kilo dog corresponds roughly to a 70 mg dose to an average man, a level relatively higher than the iron fed daily by Mitchell and Hamilton(5).

It is difficult to reconcile the final conclusion of Mitchell and Hamilton(5) that the skin and sweat glands are important excretory organs for iron with the finding that the absorption of iron occurs with difficulty and can be influenced by bodily needs. This has been amply demonstrated with radioiron in the dog(1), rat(3), and human(2) subject. The reutilization of iron released from destroyed erythrocytes following the administration of phenylhydrazine(9), transfusion of non-viable red cells(10), and normal wear and tear(11), has been shown repeatedly. The occurrence of transfusion hemosiderosis, both clinically and in normal dogs(12), indicates little or no ability on the part of humans and dogs to excrete iron. Moreover, the high tissue iron found by Finch and his co-workers(13-15) in rats and dogs fed a corn grit and lard diet is incompatible with much loss of iron from the body by any route, including the skin.

*Summary.* Following intravenous or oral administration of single doses of radioiron to dogs, little or no radioiron can be washed from the skin and hair, thus indicating that no significant amounts of iron are excreted through the skin in 1 to 7 days.

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## Some Effects of Subcutaneous Tocopherols in Normal and Tocopherol-Deficient Mice. (17718)

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Pregnant mice will resorb their fetuses when fed a tocopherol-deficient diet long enough to deplete the body stores of this vitamin; such animals, however, when treated with tocopherols will have normal litters(1,2). Although parenteral administration of tocopherols has been used effectively in preventing or repairing the histopathologic changes in voluntary muscle due to tocopherol deficiency in several species, the effect of treatment by this route on the maintenance of pregnancy has not been fully explored, due primarily to the greater effectiveness of the oral over the parenteral route of administration(3-8). To supplement

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TABLE I.
Subcutaneous Tocopherols in Normal Mice.

| Preparation | Amt, mg | Days treated | Animals | | Effect on reproductive organs |
|---|---|---|---|---|---|
| | | | No. | Sex | |
| Alpha phosphate | 15-25 | 7-65 | 8 | F | None |
| ” succinate A | 12-27 | 4-100 | 11 | M | ” |
| ” ” | 12-28 | 19-89 | 10 | F | Continuous vaginal cornification |
| ” ” | 4-8† | 25-46 | 2 | F | Continuous vaginal cornification |
| ” ” | 18-19 | 52-69 | 5 | SF | Continuous vaginal cornification |
| ” ” B | 20-27 | 30-35 | 5 | F | Initial vaginal cornification, 2 to 6 days |
| ” ” C | 20-28 | 46-50 | 5 | F | None |
| ” palmitate | 18-24 | 15-37 | 7 | F | Limited intermittent vaginal cornification |
| ” ” | 15-34 | 44-50 | 7 | M | None |
| Mixed natural concentrate, 34% | .25 cc/ daily, 4 days | 14 | 4 | F | ” |

† Pellet implanted in omentum.
SF, spayed female.

TABLE II.
Subcutaneous Tocopherols in Pregnant Tocopherol-deficient Mice.

| No. animals | Tocopherol preparation | | | Litters |
|---|---|---|---|---|
| 5 | Alpha palmitate | | 19-23 mg | Normal |
| 5 | ” phosphate | | 20-29 ” | ” |
| 5 | ” succinate (C) | | 14-26 ” | ” |
| 5 | delta phosphate | | 20-82 ” | ” |
| 6 | Mixed natural concentrate | | .05-.25 cc | ” |
| 14 | — | None | — | Dead |

the available data on the reaction of mice to tocopherols, experiments were undertaken: to determine the effect of several tocopherol preparations administered subcutaneously on the local reaction of the tissue adjacent to the site of administration in animals fed a standard, stock diet or a tocopherol-deficient diet; and to test the effectiveness of these preparations on maintenance of pregnancy during tocopherol deficiency.

*Methods.* Mice of a homogeneous, inbred strain (C57) were kept on wood shavings in individual cages and given food and water *ad libitum.* Stock animals were reared on a commercial diet (Wayne Dog Blox or Rockland Complete Rat Diet); whereas those used for tocopherol-deficiency studies were prepared by transferring the pregnant mothers from the stock to a tocopherol-deficient diet* and continuing this diet subsequently for the young. The following crystalline esters of tocopherol were made into cylindrical pellets 2 mm in diameter and 2 to 8 mm long for implantation: d-alpha-tocopheryl palmitate, phosphate, or succinate, and d-delta-tocoph-

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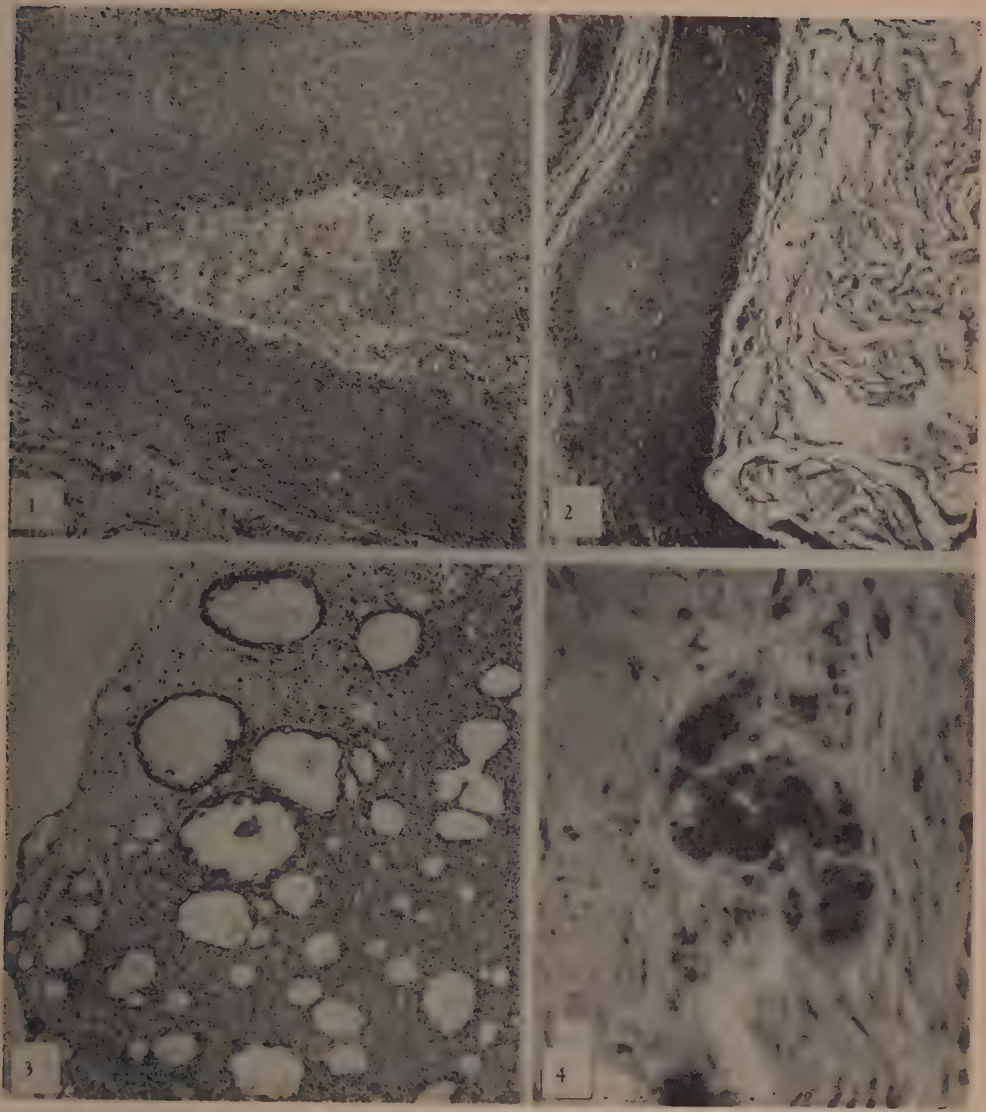
* The tocopherol-deficient diet consisted of: casein 20%, starch 50%, salts 2.5%, yeast 7.5%, lard 18.0%, cod liver oil 2.0%.



Photomicrographs of paraffin sections, 10 $\mu$. Ehrlich's hematoxylin and Kinyouin's acid-fast stains. Figs. 1-3, 67×; Fig. 4, 290×.

FIG. 1. Area of subcutaneous tissue which surrounded a pellet of alpha-tocopheryl succinate (A) in a female mouse for 52 days. Necrotic tissue is near the central pellet site which is enclosed by a connective tissue capsule and infiltrated with leucocytes.

FIG. 2. Pellet area removed from a female 28 days after implantation, showing the emulsified alpha-tocopheryl palmitate pellet (right) and connective tissue capsule with leucocytic infiltration (left).

FIG. 3. Multilocular cysts of mixed natural tocopherol solution in the subcutaneous tissue of a female for 14 days. The area is infiltrated with leucocytes and connective tissue. The tocopherol solution in the peripheral part of the cysts was acid-fast.

FIG. 4. Section from the peripheral part of the connective tissue capsule shown in Fig. 1, showing histiocytes of various size containing acid-fast pigment.

eryl phosphate. A mixed natural tocopherol solution[†] was injected. The subcutaneous tissue over the dorsal shoulder region was chosen as the site for administration in both instances. The pellets were implanted by means of a metal, tubular applicator inserted through an incision at the base of the tail and passed cephalad in the subcutaneous tissue. The tocopherol solution was injected via a tuberculin syringe and 27 gauge needle inserted through the skin and directed an inch or more from the site of entrance. Daily vaginal smears were made to determine the regularity of the estrous cycles, and to verify the time of insemination and course of gestation. The animals were killed by illuminating gas at various intervals after treatment. The tissues were fixed in Zenker-acetic, cut in paraffin at 10 $\mu$, and stained with Ehrlich's hematoxylin and Kinyouin's acid-fast stain.

*Local reaction to subcutaneous tocopherols.* Males and females (30 to 50 days of age) fed the stock commercial diet, and pregnant tocopherol-deficient females (75-125 days of age) were treated with tocopherols as shown in Tables I and II. All the tocopherol preparations produced local reactions in the subcutaneous tissue (Fig. 1-4). The alpha-tocopheryl palmitate and the concentrate of mixed natural tocopherols appeared to produce the least amount of local reaction. The alpha-tocopheryl succinate and delta-tocopheryl phosphate were more irritating, whereas the alpha-tocopheryl phosphate usually resulted in ulceration of the overlying skin and occasional loss of the pellet.

Pellets of alpha-tocopheryl succinate which could be compressed into a firm mass were removed from the implantation area, dissected free of tissue, dried in a desiccator and weighed. The other esters, which emulsified in the subcutaneous tissue, and the mixed natural tocopherol concentrate, which formed multi-locular cysts, could not be recovered with sufficient accuracy to determine the amount absorbed by the animals. Some of the tocopherol was absorbed from these sites, as indicated both by the maintenance of gestation (described below) in animals fed the tocopherol-deficient diet, and by a decrease of 1-2 mg in the weight of those pellets which could be recovered and weighed accurately.

Two other reactions to these tocopherols were noted: the presence of an acid-fast pigment in the cells of the fibrous capsule around the pellet and the prolonged vaginal cornification found after implantation of certain tocopherol pellets. A pigment, similar in its staining to that found in the adrenals of mice(9), was found adjacent to the subcutaneous tocopherol deposits. Whether this pigment was of a different nature from that found in the tissues of tocopherol-deficient animals remains to be determined.

Estrogenic activity of certain tocopherols has been claimed by certain investigators and denied by others(10-13). As shown in Table I, vaginal cornification was found after implanting some of the crystalline tocopherols.[‡] The weights and histological structure of the testes and accessory reproductive organs of the males were not affected by the various tocopherols. The various contradictory results concerning the estrogenic properties of tocopherols may be due primarily to a contaminant in the particular tocopherol preparation used. Since the mixed natural tocopherol concentrate did not produce any estrogenic effects, whereas some of the crystalline preparations obtained from the concentrate did, may have been due to some substance in the tocopherol concentrate capable of inhibiting the estrogenic material.

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[†] The author is indebted to Distillation Products, Inc., Rochester, N. Y., for the crystalline tocopherols and for the mixed natural tocopherol, concentrate solution (340 mg tocopherols/g solution: containing 170 mg of alpha-tocopherol/g and 170 mg/g of varying amounts of beta, gamma, and delta tocopherol).

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[‡] Three different preparations of *d*-alpha-tocopheryl succinate were used. The first (A) resulted in continuous vaginal cornification. Histological sections of the vaginae and uteri from the animals showed changes characteristic of estrogen stimulation. The second (B) caused vaginal cornification lasting only from the 2nd to the 6-8th day after implanting the pellets. The third (C) had no effect on the reproductive system of normal or spayed animals. This last preparation was given to the animals used for the gestation studies.

*Gestation with subcutaneous tocopherols.* To provide assurance of uniformly early and severe tocopherol depletion, the experimental animals used in this study represent offspring of mothers kept throughout pregnancy and lactation on the tocopherol-deficient diet. The young were continued on this diet and mated, when 75-100 days of age, with males reared on the stock diet. The untreated animals resorbed their young, whereas those given the tocopherol preparations during the first week of gestation had normal litters. (Table II).

*Summary and conclusions.* 1. Pellets of crystalline tocopherol esters (d-alpha-tocopheryl palmitate, phosphate, or succinate and d-delta-tocopheryl phosphate) were implanted into the subcutaneous tissue over the dorsal shoulder region of male and female mice. A solution of mixed natural tocopherols was injected into the same region in other animals.

2. All the tocopherol preparations produced local tissue changes resembling a foreign body reaction. Qualitatively, alpha-tocopheryl palmitate and mixed natural tocopherol concentrate produced slight local reaction. The reaction to alpha-tocopheryl succinate and delta-tocopheryl phosphate was greater, and that to alpha-tocopheryl phosphate the most pronounced.

3. All the tocopherol preparations maintained gestation in tocopherol-deficient mice.

4. Two of the alpha-tocopheryl succinate and the alpha-tocopheryl palmitate preparations produced intermittent or continuous vaginal cornification.

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## Turnover of $P^{32}$ in Different Types of Nuclei from Rabbit Liver.* (17719)

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Hitherto investigations into nuclear metabolism have been carried out with mixtures of nuclei isolated from the various cells which make up the tissue being studied. By differential centrifugation it has been possible to separate nuclei isolated from rabbit liver into two fractions, one made up predominantly of hepatic cell nuclei, the other containing nuclei of other types of cells present in the liver and to compare $P^{32}$-turnover in hepatic cell nuclei with other types of nuclei found in the liver.

Nuclei were isolated in 5% citric acid as previously described(1). They were then subjected to centrifugation at 900 g for 3 minutes, after which successive layers were removed with a pipette and examined microscopically. The nuclei of the upper layers were predominantly spherical, while those near the bottom of the centrifuge tube were predominantly ellipsoidal, fusiform and cylindrical, together with occasional ones which were constricted and lobed. By repeated centrifugation (5x) of the upper and lower layers, about half of the nuclei could be separated into the two groups mentioned above (Fig. 1 and 2). In each group counts were made of the frequency of the spherical and non-spherical types. In Group 1, 90-95% of the nuclei were spherical, while in group 2, 98-99% were non-spherical. On the basis of morphology, the predominant type of nucleus in Group 1 could readily be identified as that of the hepatic cell. Those of Group 2, could be recognized as fibroblast,

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* Supported by a grant from the U.S. Public Health Service.

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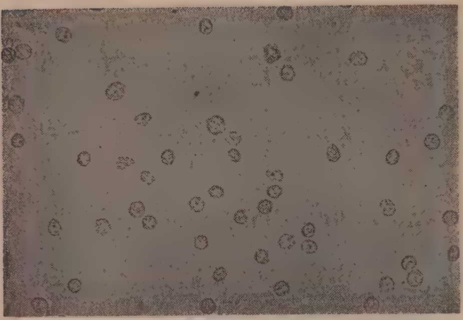

FIG. 1.
Spherical nuclei from rabbit liver.

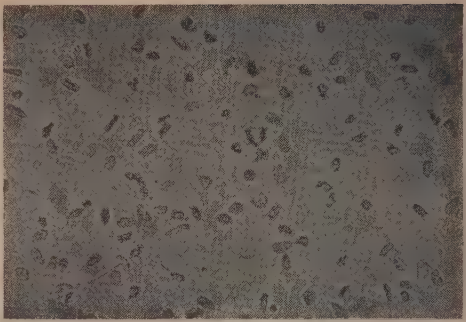

FIG. 2.
Non-spherical nuclei from rabbit liver.

TABLE I.
$P^{31}$ and $P^{32}$ in Nuclei.

| Animal | Nuclei† | Nuclei, ml | $P^{32}$,* ml | µg $P^{31}$, ml | $\frac{P^{32}}{P^{31}}$ |
|---|---|---|---|---|---|
| 1 | Spherical | $1.20 \times 10^8$ | 4352 | 93.4 | 46.6 |
| | Non-spherical | $3.28 \times 10^8$ | 8117 | 265.6 | 30.6 |
| 2 | Spherical | $1.63 \times 10^8$ | 5311 | 128.4 | 41.1 |
| | Non-spherical | $1.28 \times 10^8$ | 2979 | 92.7 | 32.1 |

* In counts per minute as of the time of injection.

† Before separation of the two types of nuclei the suspensions contained 25-33% non-spherical nuclei.

reticulo-endothelial, endothelial and polymorpholeucocyte nuclei.

$P^{32}$ was administered intravenously to rabbits as $Na_2HPO_4$, and 3 hours later the livers were perfused and the nuclei isolated and separated as described above. Total phosphorus and radioactive phosphorus were determined in aliquots of each group. Table I gives the results obtained with 2 animals.

The specific activity of the spherical nuclei from the first animal was 34% greater than that of the non-spherical nuclei, while with the second animal the difference was 23%. The total phosphorus per spherical nucleus was $7.80 \times 10^{-7}$ µg and $7.87 \times 10^{-7}$ µg in each of the animals while the values obtained for the non-spherical nuclei were $8.03 \times 10^{-7}$ µg and $7.24 \times 10^{-7}$ µg.

Since the total phosphorus content was the same in both types of nuclei within 3% the greater specific activity of the spherical nuclei could not be attributed to a lower phosphorus content per nucleus. The data therefore, indicated a higher rate of turnover of phosphorus in the spherical as compared with the non-spherical nuclei *i.e.*, in the nuclei of hepatic cells as compared with nuclei of other types of cells found in the liver. It was previously shown that most of the $P^{32}$ in liver nuclei was in the nucleic acid and that it was released from the nucleus as nucleotide phosphorus and not as inorganic phosphate(1,2). It can be concluded therefore that nucleic acid turnover in the nuclei of hepatic cells is more rapid than that of nuclei of other cells in the liver.

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# Transplantation of Cornea to Chorio-Allantoic Membrane, with Observations on Virus Inoculation. (17720)

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It has been known for many years that a number of normal and tumor tissues, derived from various species, can be grafted upon the chorioallantoic membrane (CAM) of the developing chick embryo. Such tissue grafts may remain sufficiently viable to support multiplication of certain viruses which are adapted to propagation in the transplanted tissue, but not necessarily to growth in the chick embryo. Thus, Tenbroeck has demonstrated multiplication of hog cholera virus in minced hog testicle implanted onto the CAM, although the virus had not been adapted to the chick(1). Goodpasture, Douglas and Anderson(2), and later Blank, Coriell and Scott(3) reported that intact human skin could be successfully grafted onto the CAM and that the viruses of vaccinia, variola and herpes simplex, which are infectious for the chick embryo, readily produced typical inclusion bodies in the epithelial cells of the transplant. Similar results were obtained, on occasion, with the virus of herpes zoster which has not as yet been grown in the embryonated egg(3,4). Finally, grafts of fetal human membranes onto the CAM have been found to support the viruses of herpes simplex, variola, vaccinia and mare abortion(5).

It was thought that the transplant technic might prove to be of value in the study of viral agents affecting human ocular tissues, some of which have not as yet been adapted to propagation in host cells other than those of man. To explore this possibility attempts were made to graft corneal tissue of rabbit and man onto the CAM. The results are briefly summarized below.

*Methods and materials.* Embryonated hen's eggs of various ages were used. In the case of eggs 8-10 days of age the shell over the natural air sac was removed and a small slit was made in the shell membrane by means of a sterile hypodermic needle. Through this needle a drop of saline was deposited which seeped through the opening under the shell membrane, as a result of which the latter could easily be peeled off the underlying CAM. In embryos older than 10 days the CAM was exposed by means of a false air sac. Rabbits were sacrificed and their eyes enucleated as soon after death as possible. On occasion, however, the eyes were removed from rabbits stored at 4°C for as long as 48 hours after death. The enucleated eyes were kept for 1 - 14 hours in phosphate-buffered saline solution of pH 7.2 containing 500 units of penicillin and 0.1 mg of streptomycin per ml. The eyes of a few human stillborn infants were enucleated and kept in the same manner. For the transplanting the cornea was cut off at the limbus with corneal scissors and spread on a sterile cork disk with the epithelium facing downward. In most experiments the inner surface of the cornea was scraped with a fine scalpel in an attempt to remove Descemet's membrane. For infection with virus the epithelial side of the cornea was scarified either by means of a sterile scalpel or by multiple pin pricks with a sterile 24 gauge hypodermic needle. Virus was then rubbed onto the prepared epithelium with a cotton swab. The normal or infected corneae were then lifted off the cork with a sterile forceps and transferred onto the CAM with the endothelial side facing the membrane, preferably over a large

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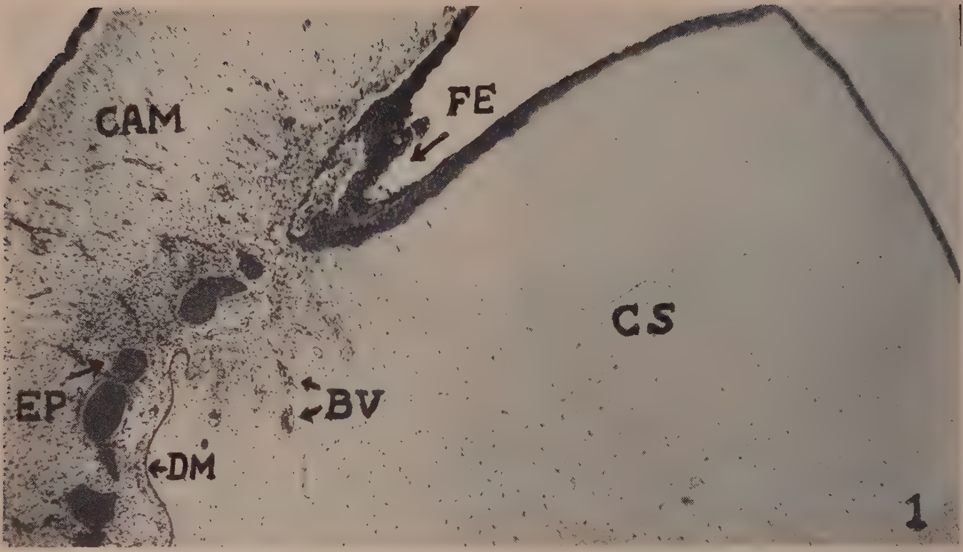

FIG. 1.
Rabbit cornea 72 hours after grafting onto the CAM of the chick embryo. Fusion is apparent in the area where Descemet's membrane ends abruptly. CS—corneal stroma, FE—fusion of epithelium of cornea with epithelium of CAM, DM—Descemet's membrane, EP—epithelial pearls, BV—blood vessels of the chick invading corneal stroma, CAM—chorio-allantoic membrane. (H & E × 50).

blood vessel. The eggs were then sealed with two layers of scotch tape and incubated at 37°C. At various intervals thereafter the cornea with the adherent underlying CAM was removed, fixed, sectioned and stained with hematoxylin and eosin.

The viruses of vaccinia and herpes simplex were used in these tests. The former was obtained from a commercial source as calf lymph and passed once on the CAM; The latter had been adapted by passage through hen's eggs and was used in the form of a 10% suspension of allantoic membrane infected with the HF strain.

*Observations and results.* In earlier experiments with corneal tissue of the rabbit no effort was made to remove Descemet's membrane. Under those circumstances the graft failed to become attached to the CAM, it increased in thickness, and presented an opaque, yellow gray appearance. It became necrotic in a few days. However, in one experiment the cut surface of the cornea happened to be in apposition with the CAM and in that case fusion occurred in the small area

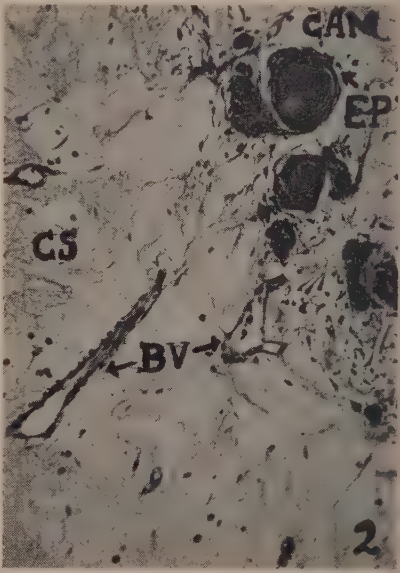

FIG. 2.
Area of invasion of chick blood vessels into rabbit cornea 8 days after grafting. CAM—chorio-allantoic membrane, EP—epithelial pearls, CS—corneal stroma, BV—blood vessels. (H & E × 250.)

of apposition (Fig. 1). This observation led to attempts to remove Descemet's membrane before grafting the cornea onto the CAM. Under these conditions fusion was obtained regularly. The cornea appeared swollen, bluish and opaque and could not be dislodged from the CAM. On histological examination it was found that the epithelium of the CAM disappeared rather rapidly except for remnants in form of epithelial pearls. The corneal stroma showed marked separation of the lamellae with an increase of tissue fluid. This change was apparent within 24 hours. After 48 hours polymorphonuclear cells of the chick were found between the lamellae. Blood vessels were seen to invade the stroma from the mesodermal layer of the CAM where Descemet's membrane had been removed (Fig. 2). Where removal was incomplete the membrane was seen to form a firm barrier against the invading blood vessels and leucocytes (Fig. 3). Vascularization of the stroma was noted after 48 hours of incubation and was observed to extend to Bowman's membrane by the 8th day. The corneal epithelium, as a rule, was well preserved (Fig. 4), however, on occasion the superficial cells were cast off and the basal layers showed vacuolization of the

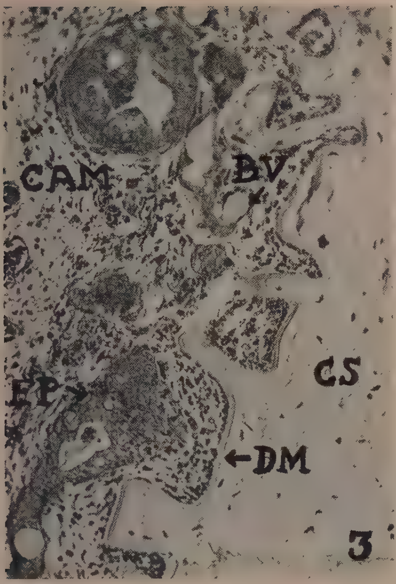

FIG. 3.

Rabbit cornea 9 days after grafting. Descemet's membrane is thrown into folds and forms a firm barrier against invading vessels. Epithelial pearls and newly formed blood vessels appear in the area of apposition. CS—corneal stroma, DM—Descemet's membrane, BV—blood vessels of chick, EP — epithelial pearls, CAM — chorio-allantoic membrane. (H & E × 111.)

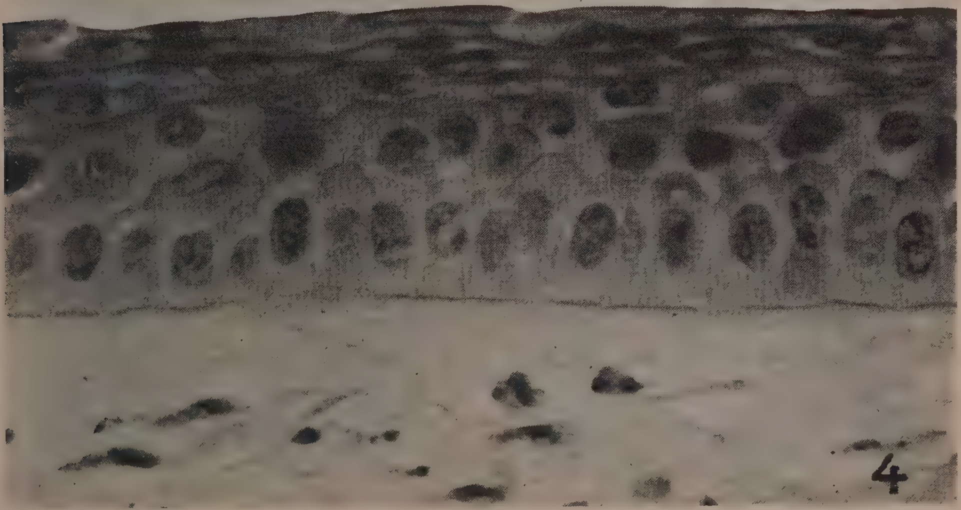

FIG. 4.

Rabbit cornea 8 days after grafting onto CAM showing well preserved epithelium. (H & E × 1,000.)

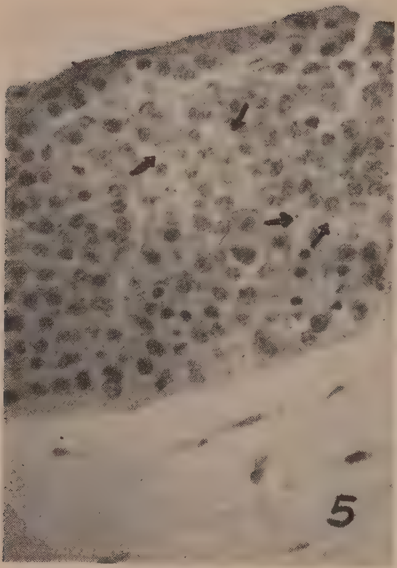

FIG. 5.
Proliferated epithelium of corneal graft 72 hours after infection with vaccinia virus by the needle prick method. Numerous cytoplasmic inclusion bodies marked by arrows. (H & E × 290.)

nuclei. Proliferation was indicated by not infrequent mitotic figures. Viability of the epithelium was further proven by the appearance of characteristic cytoplasmic inclusions following infection with vaccinia virus (Figs. 5,6) and, in cases of infection with herpes simplex, of intranuclear inclusions (Fig. 7).

Since the experimental period was necessarily limited to 8-10 days, attempts were made to preserve viable corneae for longer periods by re-grafting onto a second chick embryo. Successful takes were obtained but the corneal epithelium was shed in most grafts. In only one instance of infection with vaccinia virus on second transplant was the epithelium well enough preserved to permit the demonstration of inclusion bodies.

Experiments with human corneal tissue were limited to only three cases, two stillborn infants and one premature fetus. All 3 were partially macerated. One cornea of one of these showed satisfactory fusion. These studies will be continued.

*Summary and conclusions.* 1. Human and

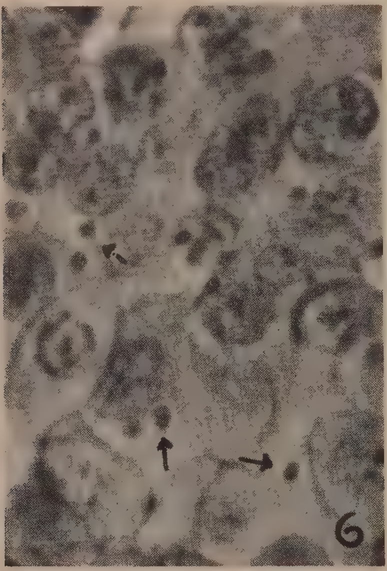

FIG. 6.
Enlarged view of corneal epithelium shown in Fig. 5. Numerous cytoplasmic inclusion bodies. (H & E × 1360.)

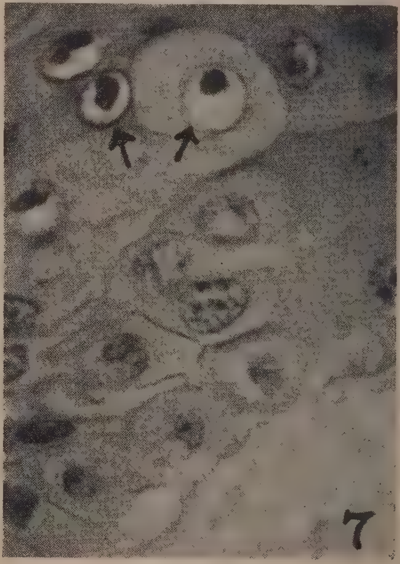

FIG. 7.
Rabbit corneal graft 72 hours after infection with herpes simplex virus showing intranuclear inclusion bodies. (H & E × 1,000.)

rabbit cornea may be grafted onto the chorioallantoic membrane of the chick embryo and kept viable.

2. When the graft is successful, it presents a bluish appearance and is firmly adherent to the membrane. Histologically, such grafts are seen to be invaded by chicken blood vessels into the normally avascular stroma, and show preservation and proliferation of the corneal epithelium.

3. The viruses of vaccinia and herpes simplex were found to give rise to inclusion bodies within 24-72 hours in the epithelial cells of the corneal graft.

4. Serial passages of corneal grafts were complicated by the frequent loss of the epithelium and in only one case were inclusion bodies found after the inoculation during the second grafting.

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## Transformation Type Specificity of *H. influenzae*.* (17721)

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Avery, MacLeod and McCarty(1) isolated from type III *D. pneumoniae* a desoxyribonucleic acid which in a suitable environment endowed R cells, derived from type II *D. pneumoniae*, with characteristics of S cells of type III. The transformed cells became encapsulated, elaborated the specific carbohydrate of type III and exhibited colony traits of this type. McCarty and Avery later reported an improved method for isolating specific pneumococcus desoxyribonucleic acids which they applied to types II, III and VI(2). Boivin and others(3) and Weil and Binder(4) reported the very irregular occurrence of transformations of *E. coli* and *S. dysenteriae* in the presence of crude desoxyribonucleic acid containing extracts made from the specific type into which the R variant was changed. This paper reports 1) the isolation from type b *H. influenzae* of a desoxyribonucleic acid which transforms an R strain Rbl, derived from type b *H. influenzae*, to type b cells during an 18 to 24 hour period of growth and 2) the isolation from type c *H. influenzae* of an unpurified transforming principle which endows the same R strain, Rbl, with properties of type c *H. influenzae*.

*Material and Methods.* Isolation of transforming principles. The desoxyribonucleic acid of type b *H. influenzae* was isolated and purified by the method which McCarty and Avery(2) applied to pneumococcus. Type b *H. influenzae* grown for 6 to 7 hours on Levinthal agar plates was harvested by washing each plate with 4 cc of a solution containing 0.1 M sodium chloride and 0.1 M sodium citrate. After 2 washings in this fluid the yield from 300 plates was reduced by centrifugation to a volume of 40 to 50 cc and frozen in carbon dioxide ice. After thawing at 4°C the volume was made up to 500 cc with the citrate saline solution and 5 cc of 10% sodium desoxycholate was then added; marked dissolution of the organisms occurs within 30 minutes at room temperature, the creamy appearing suspension clearing almost completely. Three extractions with chloroform and amyl alcohol were carried out to remove protein. The addition

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* The work in this communication was supported by a Commonwealth Fund grant.

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of 2 volumes of absolute ethyl alcohol to the partially deproteinized solution yielded a fibrous precipitate which was allowed to remain in the alcohol at 4°C overnight. The fibrous precipitate drained of alcohol and redissolved in 0.85% sodium chloride pH 7.0 was extracted twice more with chloroform and amyl alcohol, again precipitated with 2 volumes of alcohol, washed in absolute alcohol and redissolved in physiologic saline. Ribonucleic acid was eliminated by digestion with ribonuclease, and the desoxyribonucleic acid was separated from the specific polysaccharide of type b by precipitation in the presence of 0.1 volume of 10% calcium chloride and 0.2 volume of absolute alcohol. The final precipitate was dissolved in 20 cc of 0.85% saline. This solution formed no precipitate with type b specific rabbit antiserum, but showed positive reactions for desoxyribonucleic acid when examined by the diphenylamine(5) and Stumpf (6) tests. We are indebted to Dr. Zacharias Dische for these last determinations. The isolation of unpurified type c *H. influenzae* transforming substance has been carried out by a process identical with that used for type b up to the point of digestion with ribonuclease. The type c substance used in the experiments to be reported is therefore a crude product and contains the specific polysaccharide of type c. The final fibrous precipitate obtained from the growth on 100 plates was dissolved in 25 cc of physiologic saline, pH 7.0.

R strain used for transformation: The R strain of *H. influenzae,* Rbl, used in these experiments was derived from a strain of type b *H. influenzae* by selection of a spontaneously appearing, non-iridescent, non-opaque colony from among the iridescent, opaque ones characteristic of S cells after growth on Levinthal agar for 18 hours; subculture and isolation of characteristic R colonies have been repeated many times. Cultures of this Rbl strain have been used for various purposes in our laboratory for 6 years, and at no time has evidence of type specificity been detectable by study of colony type, capsular swelling tests, or precipitin reactions on supernatants of broth cultures. Twenty passages of this strain through mice (in mucin suspensions) failed to demonstrate presence of type specific organisms. Anti-R *H. influenzae* antiserum: Rabbits were injected intravenously with gradually increasing volumes of a suspension of Rbl cells of *H. influenzae* (approximately 1.5 to 2.0 billion cells per cc) in 0.2% formalinized saline. An initial dose of 0.1 cc was increased daily by 0.1 cc for 4 days of each week until 1.0 cc daily was reached. Serum of rabbits injected for a period of at least 2 weeks has provided a suitable environment when used in a 1:50 dilution in the presence of desoxyribonucleic acid under the circumstances of the experiments described. The antiserum was heated at 60°C for 30 minutes.

*Experimental.* Transformation of non-typable, R *H. influenzae* strain, Rbl, to type specific cells: The environmental constituents found to be essential for the transformation of pneumococci by their specific desoxyribonucleic acids(7) proved adequate for transformation of *H. influenzae*. These constituents could be supplied by the addition of whole anti-Rbl rabbit antiserum or by the addition of the 3 separate factors found to be responsible for its transforming activity —1) R agglutinins, furnished by the gamma globulin fraction of anti-Rbl serum prepared by method of Taylor(8), 2) a protein substance, furnished by bovine serum albumin fraction V† and 3) sodium pyrophosphate, which restores a function lost by dialysis of rabbit anti-R antiserum. These 2 varieties of environmental influence—1) whole rabbit anti-R *H. influenzae* antiserum or 2) the 3 separate components—were examined for their effect in a system containing a transforming principle of *H. influenzae,* either

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† Prepared by Armour and Co. according to techniques developed in the laboratory of Dr. E. J. Cohn.

TABLE I.
Transformation of Rbl Cells of *H. influenzae* into Specific Type b and Type c *H. influenzae*.

| Environment | | Transformation Quintuplicate tests 1 through 5 |
|---|---|---|
| Levinthal broth plus | Transforming principle | |
| Whole rabbit anti-Rbl *H. influenzae* antiserum dilution 1:50 | 0 | R only |
| | DNAb | $S^b$ + R |
| | type c unpurified | $S^c$ + R |
| Gamma globulin from anti-Rbl *H. influenzae* antiserum + bovine albumen fraction V + Na pyrophosphate | 0 | R only |
| | DNAb | $S^b$ + R |
| | type c unpurified | $S^c$ + R |

DNAb—Desoxyribonucleic acid of type b *H. influenzae.*
$S^b$—Type b *H. influenzae.*
$S^c$—Type c *H. influenzae.*

type b or type c, and a representative population of young Rbl *H. inflenzae* cells. The nutrient medium, Levinthal broth, which provided R cell growth during which transformation occurred, had not been adsorbed with charcoal.

Transformation of R strain Rbl to type b *H. influenzae*: The circumstances under which transformation of R cells to S cells of type b *H. influenzae* occurred are shown in Table I. It is seen that when type b desoxyribonucleic acid (0.1 cc of 1:10 dilution) is present in an environment containing approximately 1,000,000 cells (a 2 mm loopful of a 6 hour culture) of R *H. influenzae* in 2 cc of Levinthal broth containing 1:50 dilution of anti-R *H. influenzae* rabbit serum, 5 samples after incubation for 18 to 24 hours showed presence of S cells of type b *H. influenzae.* The change to encapsulated type specific cells was evidenced by capsular swelling with type specific antiserum and formation of iridescent, opaque colonies on Levinthal agar. This transformation occurred also in the presence of the 3 separate constituents of anti-R serum, chosen by analogy with comparable experiments in conversion of R to S pneumocococci.

Transformation of R strain, Rbl, to type c *H. influenzae*: When an unpurified transforming principle isolated from type c *H. influenzae* was added in 0.1 cc quantities of a 1:10 dilution to environments identical with those described above, the R cells Rbl were transformed into type c *H. influenzae*; the transformed cells showed capsular swelling when exposed to type c specific antiserum, and opaque colonies exhibiting iridescence appeared after growth for 18 hours on Levinthal agar. The transformation in all of the quintuplicate tests are also shown in Table I.

*Summary.* The data provide another example of the control of the genetic constitution of microorganisms by specifically oriented desoxyribonucleic acids. An R strain, Rbl, derived from type b *H. influenzae* was endowed with properties of type b under the influence of a desoxyribonucleic acid isolated from type b *H. influenzae.* This same R strain, Rbl, was transformed to type c *H. influenzae* when grown in an appropriate environment in the presence of an unpurified transforming principle isolated from type c *H. influenzae.*

Rbl has also been transformed to Sa and Sd. The transforming activity of desoxyribonucleic acid has been shown to be destroyed by a crystalline desoxyribonuclease supplied by Dr. Maclyn McCarty.

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# Gastric Secretion of Iodide at Low Serum Iodide Levels. (17722)

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Several reports in the literature show that the concentration of iodide in gastric juice may exceed concurrent plasma iodide levels many fold(1,2,3,4). Davenport(5), in an attempt to elucidate the mechanism of this phenomenon, found an inverse curvilinear relationship between the concentration of iodide in gastric juice and the plasma iodide level, when the latter was in the range of 0.1 to 3.0 millimol per liter. No correlation between the concentration of iodide in the gastric juice and other variables of gastric secretion was observed. Since pouch dogs were used in these studies and gastric secretion was stimulated with histamine, one must presume that the secretion studied was consistently in the acid pH range.

The experiments reported here extend the observations of Davenport to the gastric excretion of iodide at consistently low serum iodide levels, and to its secretion in alkaline gastric juice. It was hoped that under these conditions a correlation between the gastric excretion of iodide and other variables of gastric secretion would become apparent. These expectations were, at least in part, fulfilled. A study of the iodide concentration in alkaline gastric juice was made possible by the use of a preparation recently described by Morton and Stavraky(6). The use of radioactive iodine as a tracer made it feasible to study the gastric excretion of iodide at low serum iodide levels; by means of the isotope the injected iodide could be distinguished readily and in small amounts from the iodine normally present in the body.

*Methods.* The gastric excretion of radioiodide was studied on 14 dogs. Individual experiments lasted from 2½ to 13 hours. Three dogs had total gastric pouches with an intact neurovascular supply, and 11 dogs were used in acute experiments. In the latter the stomach of the anesthetized animal was exposed surgically and severed from the duodenum and esophagus. Secretion of acid gastric juice was stimulated by food, or by intravenous or subcutaneous injection of histamine, insulin, or pilocarpine; alkaline gastric juice was evoked by stimulation of the greater curvature through arterial injection of acetylcholine(6). The animals were given a single intravenous injection of 20 to 200 microcuries of radioiodide,† with enough ordinary sodium iodide to raise the initial blood level to $10^{-2}$ to $10^{-1}$ millimol per liter. The carrier iodide was added in order to minimize sources of experimental error which may be caused by adsorption of the isotope on the equipment used, and to stabilize the serum iodide levels. One hundred microcuries of iodide consisting only of the isotope $I^{131}$ weigh about 8 x $10^{-7}$ mg(7). Presumably, this amount of inorganic iodide would disappear rapidly from the serum through mechanisms such as removal by the thyroid or conversion to organic iodo compounds(8,9). Chemical determinations and

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radioactivity assays were done on individual fractions collected generally over 30-minute periods. Blood samples were withdrawn by venipuncture during the first half hour after the injection of radioiodide, at the end of the experiment, and at various intermediate periods. For radioactivity measurements, 0.2 ml of the liquid to be assayed was evaporated on an aluminum planchet. Sodium bicarbonate was added to the gastric juice samples to neutralize free acid and to raise their solid content to that of the serum. An end window tube was used for counting and the customary corrections were applied. The assays were reproducible to within ±10%, so that the biological variations encountered exceeded the error involved in the assays by a factor of 10. The iodide levels in gastric juice and serum were calculated on the assumption that the specific activity of the iodide in the serum and gastric juice was the same as that of the administered iodide(9). To the extent that the body iodide may have diluted the administered labeled iodide, the iodide levels thus calculated will be low.

*Results and Discussion.* We found iodide to be concentrated in gastric secretions with pH values ranging from 1 to 8, and irrespective of the secretagogue employed. In 2 experiments, in which an alkaline secretion was produced initially with acetylcholine and then changed to an acid secretion by injection of histamine, the isotope level in the gastric juice remained appreciably higher than the isotope level in the serum throughout with no significant change coincident with the fall in pH. Within individual experiments as well as among different experiments the gastric juice iodide levels showed wide fluctuations. We believe that these fluctuations may, at least in part, be due to wash out effects. Gastric juice levels considerably in excess of the serum levels persisted even after several hours of continuous secretion, and there was no fall in the concentration ratios at the end of prolonged experiments. Therefore, the concentration phenomenon cannot be ascribed to an initial high tide of radioiodide in the serum, which manifests itself in the gastric secretion later on.

The most significant results of our study are shown graphically in Fig. 1. Plotted on the ordinate are the values for iodide concentrations in the samples of gastric juice, divided by the concurrent iodide concentrations in the serum (concentration ratios). Plotted on the abscissa are the corresponding rates of secretion, in ml/30 min./kilo body weight of the dogs. The data were obtained from 226 specimens of gastric juice with varying pH values, collected in 14 experiments. Different methods of stimulation seemed to influence the secretion of iodide only to the extent that they affected the rate of flow of gastric juice. However, only samples secreted after the first half hour following the injection of iodide are included in the graph, because in most experiments the iodide level in the gastric juice secreted during the first half hour was lower than in subsequent specimens. It is evident from the graph that the concentration ratios of iodide for gastric juice, at constant low serum iodide levels, do bear a relationship to the rate of gastric secretion. High concentration ratios were generally obtained at low rates of secretion and the opposite also held true. Thus our findings differ from the statement of Davenport that "the concentration (of iodide) in the gastric juice is independent of the rate of secretion of the juice." The distribution of the data on the graph suggests that the relationship between the concentration ratios and the rates of secretion may follow an equation of the type xy = constant. This would imply that the amount of iodide which enters the gastric juice per unit time, at constant serum iodide levels, is constant.

Our incidental observations on the dependency of the gastric juice iodide level on the serum iodide level tend to confirm Davenport's findings. However, in individual experiments a drop of as much as 50% in the serum iodide level was not necessarily accompanied by a corresponding drop of the gastric juice iodide level. Only when experiments were compared in which the serum iodide levels differed by a factor of 5 or more

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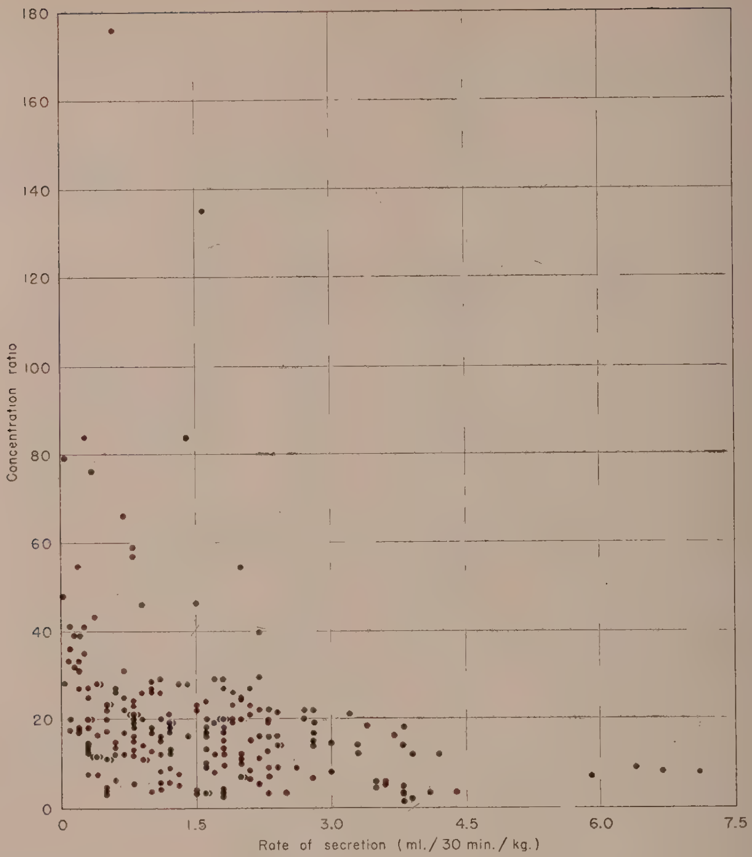

FIG. 1.

Relationship between concentration ratios of iodide for gastric juice and rates of gastric secretion.

did it become evident that the higher serum iodide levels resulted in an elevation of the gastric juice iodide levels. As was mentioned above, about half an hour was required at the beginning of most experiments before the initial rise in the gastric juice iodide leveled off. This may indicate that at least under our experimental conditions, about one-half hour is required before the serum iodide level manifests itself in the gastric juice. We have therefore recalculated some of the concentration ratios using serum iodide levels which prevailed approximately one-half hour prior to the collection of the gastric juice sample. The concentration ratios calculated in this manner are somewhat lower than the corresponding ratios given in the graph. However, since the serum iodide levels fell off rather slowly and uniformly, the distribution pattern of the values in the graph is not changed significantly. In any case it appears that a steady state between the iodide levels in the gastric juice and serum was never established. This is indicated by the comparatively slow rise of the iodide level in the gastric juice following intravenous injection, by marked fluctuations in gastric juice levels seen during the present study, and by the finding of Eisenman(10) that iodide enters the body fluids from the gastric contents very slowly.

No evidence was found to indicate that

radioiodine was present in the gastric juice in any form other than inorganic iodide. The isotope could be removed quantitatively from the secretion as palladium iodide, and paper partition chromatography(11) showed that the radioactive moiety in the secretion had the same $R_f$ value as inorganic iodide. Extensive studies on the chemical forms in which radioiodine may be present in the serum have been carried out by other workers(12,13,14). It may be presumed that the isotope was present largely as inorganic iodide in the serum in our experiments. Any physicochemical binding of the injected iodide to components of the serum, or any conversion to organic iodo compounds, would render the serum iodide concentrations calculated from the radioactivity levels fictitiously high, and the corresponding concentration ratios fictitiously low. Corrections for the difference in the total solid content of gastric juice and serum would have the opposite effect, but would operate to reduce the concentration ratios by only about 10%.

The fact that iodine was concentrated in alkaline as well as acid gastric juice suggests that the secretion of acid by the parietal cells may not be necessary to bring about the concentration phenomenon. In an experiment on a dog with 4 divided pouches (prepyloric, lower body, upper body and fundus) iodide was found to be concentrated to about the same extent in the secretion from each pouch. Reports in the literature show that iodide is concentrated in gastric juice even in the presence of severe pathological changes in the gastric mucosa, such as linitis plastica(15) or carcinoma of the body of the stomach(16,17,18). Thus it appears that iodide can be concentrated from any region of the stomach, and that the concentration effect can persist even when the mucosa is physiologically and morphologically abnormal.

*Summary*. The secretion of iodide in acid and alkaline gastric juice at low serum iodide levels was studied in pouch dogs using radioiodide as a tracer. The concentration of iodide in the gastric juice was found to be independent of the pH of the gastric secretion, but it was influenced by the rate of gastric secretion. High concentration ratios for iodide were obtained at low rates of secretion and low concentration ratios at high rates of secretion.

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# Subacute Toxicity of Radioactive Phosphorus as Related to the Composition of the Diet.* (17723)

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The study of the toxicity of radioactive isotopes becomes more and more important with the increasing use of these substances both as a research tool and as a therapeutic agent. While the mechanism of the injurious action of radiation on living cells is probably the same for external and internal radiation sources, one may expect that, from the practical viewpoint, the effects of the introduction of isotopic compounds within the body will vary in relation to the physical characteristics of the isotope (such as the nature of the radiation, its energy and rate of decay) and to the behavior in the organism of the compound containing the radioactive atom (such as its absorption, distribution, intermediary metabolism and rate of excretion). Accordingly, conditions which enhance or attenuate the injurious effects of one isotope or isotopic compound, may be ineffective if a different radioactive material is used. It is thus apparent that the toxicity, as well as the factors which may modify it, should be investigated separately for each isotope and for each isotopic compound.

In the present investigation the subacute toxicity of $P^{32}$, given parenterally to mice (in one dose and in the form of $Na_2HPO_4$) has been studied in relation to the possibility that changes in the composition of diet may modify the action of the radioactive isotope. To our knowledge, the only data on the toxicity of $P^{32}$ are those of Anthony and Snyder (1). On the basis of a few experiments on mice (presumably maintained on a stock diet), these authors suggest that the $LD_{50}$ after one injection of $P^{32}$ is around 4 microcuries per g of body weight. With this dose 4 out of 6 mice died between 16 and 21 days.

TABLE I.
Composition of Experimental Diets (% of the dry weight).

| Diet No.* | 34 | 29 | 31 | 35 |
|---|---|---|---|---|
| Casein | 10 | 25 | 10 | 25 |
| Cod Liver Oil | 1 | 1 | 2 | 2 |
| Crisco | 4 | 4 | 30 | 30 |
| Dextrin | 40 | 32 | 26 | 19 |
| Sucrose | 39 | 32 | 26 | 18 |

* In addition to the above components, the diets contained 2% Ruffex and a Salt Mixture (usually 4% of U.S.P. XII vitamin test mixture). A mixture of several B vitamins (Thiamine HCl 0.5 mg per 100 g of diet, Riboflavin 0.5, Pyridoxine HCl 0.5, Nicotinic acid 1.5, Ca pantothenate 2, Inositol 2, Paraaminobenzoic acid 2) was incorporated daily in the diets.

*Experimental.* Male mice (Rockland "all purpose" strain of approximately 20 g body weight) were transferred from a stock diet (Rockland Farms) to one of the experimental diets, the composition of which is recorded in Table I. The animals were divided in groups of 6, and each group kept in a metallic cage with raised screen floor. Water was given *ad libitum*. After 5 days on the experimental diet, the mice were injected intraperitoneally with a single dose (0.2-0.3 cc) of $Na_2HPO_4$ containing $P^{32}$ (usually in the concentration of about 1 millicurie for 0.02 mg of P). The mice were then maintained on the same diet for 21 more days, during which the food consumption was recorded daily and the animals weighed at 4-5 day intervals. In each experiment, 6 to 12 groups of mice on

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TABLE II.
Effects of Various Doses of $P^{32}$ in Mice on a Low Protein, High Fat Diet (Diet 31).

| $P^{32}$ injected, μc/g | No. of mice | Avg change in body wt, g | Time of 50% deaths, days | % of survivors at the 21st day | Avg time of survival,* days |
|---|---|---|---|---|---|
| 0 | 7 | —1.8 | >21 | 100 | >21 |
| 2 | 6 | +1.0 | >21 | 100 | >21 |
| 4 | 15 | +2.5 | >21 | 67 | 19 ± 0.8 |
| 6 | 39 | —2.5 | 14 | 20 | 15 ± 0.6 |
| 8 | 23 | —3.6 | 11 | 7 | 13 ± 0.8 |

* The values preceded by ± are the standard errors of the means.

3 to 6 different diets were used. As far as possible, the effects of changes in the composition of the diet were studied comparatively on groups run at the same time and injected with the same batch of isotopic phosphate.† As an indication of the effects of the isotope, the following criteria were used: (a) Time in days at which 50% deaths occurred; (b) Percentage of survivors at the 21st day; and (c) Average time of survival in days. Since in computing the averages a survival time of 21 days was used for the mice still alive at the end of the experiment, the figures are mostly lower than the true average survival times. In evaluating the results statistically, the probability *P* for a chance occurrence of the observed differences has been calculated, using the method of the $\chi^2$ for the percentage of survivors and the *t* test of significance for the differences in the average survival times (3).

*Results. Determination of the $LD_{50}$ dose.* The effect of increasing amounts of $P^{32}$ introduced in mice maintained on a low protein, high fat diet (Diet 31) are shown in Table II. The differences between the groups injected with 4 and 6 microcuries of $P^{32}$ per g are statistically significant ($P < 0.01$). It is apparent that the $LD_{50}$ dose for mice on Diet 31 and for experiments of 21 days duration lies between 4 and 6 microcuries per g of body weight.

*Fatty infiltration of the liver and lipotropic factors in the diet.* It seemed possible that anatomic or functional alterations of the liver, such as those accompanying the fatty infiltration of this organ, might lower the resistance of the animals to the radiation injury. Accordingly, various doses of $P^{32}$ (2-8 microcuries per g) were given to 35 mice on Diet 31 (which was high in fat and low in protein, *e.g.* deficient in lipotropic factors). The results were compared with those observed in 43 mice maintained on the same diet, supplemented with choline chloride (0.5%).‡ In other experiments, prior to the introduction of $P^{32}$ 20 mice on Diet 31 received 2 or 3 injections of 0.10-0.15 $CCl_4$, as indicated by Barrett *et al.*(5), 10 other mice were treated identically except that choline chloride was

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† This method of comparison should minimize the errors caused by variations in the conditions of the experiments (such as the season, room temperature, etc.) and in the concentration and chemical form of the $P^{32}$ injected. The data on the radioactivity of the samples, supplied by the Oak Ridge National Laboratories with each shipment of $P^{32}$, were usually checked by us against a calibrated uranium source. However, only recently have we been aware of the possibility that some samples might have contained $P^{32}$ in the form of acid-labile esters or pyrophosphate(2). This possibility could perhaps explain certain differences between the results of experiments, made under apparently identical conditions, but in which different batches of $P^{32}$ had been used.

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‡ On the basis of the data for the body weight and food consumption, the amounts of choline chloride ingested by our mice on the choline supplemented diets were 3 or 4 times greater than those which have been found adequate for complete protection against both fatty liver and renal damage of young rats on low protein, high fat diets(4).

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TABLE III.
Effect of Varying the Protein and Fat Content n the Diet of Mice Injected with $P^{32}$ (6 μc per g).

| Diet No. | No. of mice | Food consumption, g/day | Change in body wt, g | Time of 50% deaths, days | % of survivors at 21st day | Avg time of survival,* days |
|---|---|---|---|---|---|---|
| 34 (low fat, low protein) | 80 | 3.6 | —0.9 | 16 | 40 | 16 ± 0.4 |
| 31 (high fat, low protein) | 39 | 1.7 | —2.5 | 14 | 20 | 15 ± 0.6 |
| 29 (low fat, high protein) | 37 | 3.6 | +3.9 | 13 | 24 | 14 ± 0.8 |
| 35 (high fat, high protein) | 70 | 2.4 | +3.3 | 12 | 3 | 12 ± 0.2 |
| Controls (Diet 35, not inj. with $P^{32}$) | 11 | 27 | +4.2 | >21 | 100 | >21 |

* The values preceded by ± are the standard errors of the means.

TABLE IV.
Effect of Increases in the Phosphate Content of the Diet of Mice Injected with $P^{32}$ (6 μc/g)

| Series of exp. and diet No.* | No. of mice | Phosphate in diet (as $H_3PO_4$) % | Food consumption, g/day | Change in body wt, g | Time of 50% deaths, days | % of survivors at 21st day | Avg time of survival,† days |
|---|---|---|---|---|---|---|---|
| I (35)‡ | 24 | 1.3 | 2.5 | +3.5 | 12 | 8 | 12 ± 0.6 |
| (35)§ | 25 | 2.9 | 2.4 | +2.1 | 13 | 28 | 14 ± 1.2 |
| II (34)‡ | 53 | 1.3 | 3.7 | —0.1 | 16 | 40 | 16 ± 0.5 |
| (34)¶ | 29 | 3.2 | 4.1 | +0.6 | >21 | 64 | 18 ± 0.7 |
| III (34)** | 18 | 0.3 | 2.9 | —4.0 | 14 | 11 | 15 ± 0.7 |
| (34)†† | 18 | 2.9 | 3.2 | —2.5 | >21 | 61 | 19 ± 0.6 |

* No. of diet in parentheses.

† The values preceded by ± are the standard errors of the means.

‡ Salt mixture U.S.P. XI, 4 g % of diet.

§ Salt mixture U.S.P. XI, 3 g, $K_2HPO_4$ 1.95 g, $NaH_2PO_4$ 0.7 g, NaCl 0.35 g % of diet.

¶ Salt mixture U.S.P. XI 2 g, $K_2HPO_4$ 2.6, $NaH_2PO_4$ 0.9, NaCl 0.5 g % of diet.

** Salt mixture U.S.P. XI 1 g, KCl 1 g, NaCl 1 g, Ca lactate 0.8 g, $MgSO_4$ 0.2 g % of diet.

†† Salt mixture U.S.P. XI 1 g, $K_2HPO_4$ 1.95 g, $NaH_2PO_4$ 0.7 g, NaCl 1.35, Ca lactate 0.8 g, $MgSO_4$ 0.2 g % of diet.

added to the diet. From the data (which are omitted for brevity) no difference was detectable between the groups on either the choline deficient or the choline supplemented diet, whether or not poisoned with $CCl_4$.

*Variations in the level of dietary fat and protein.* Table III shows the results obtained in several experiments, in which the mice were maintained on 4 different diets, respectively low in protein and fat (Diet 34), high in protein and low in fat (Diet 29), low in protein and high in fat (Diet 31), high in both protein and fat (Diet 35). It is apparent that the susceptibility to the injurious action of $P^{32}$ increased distinctly with the increase in the level of protein, or fat, or both. All differences in the values were highly significant ($P<0.01$), except for the difference between Diets 34 and 31 (which were not significant) and between Diets 34 and 29 (which were moderately significant for the average survival times only). During the 21 days after the injection of $P^{32}$, the average body weight of the mice on the low protein diets decreased, while it increased in the animals on the high protein diets. However, both decreases and increases in weight were not marked (less than 20% of the initial weight), and no correlation is apparent between these changes and the data on the toxicity of $P^{32}$.

*Variations in the phosphate content of the diet.* It was thought that by increasing the amount of phosphate ingested, the exchange between the $P^{32}$ initially deposited in the bone and the non-isotopic phosphate circulating in the plasma might be enhanced, and that the increased rate of mobilization of $P^{32}$ would shorten the effective time of exposure of the tissues, especially the bone marrow. Table IV shows that both the percentages and the times of survival were greater in the mice on phosphate enriched diets. The differences were quite significant in the II and III series of experiments ($P<0.01$, except for the average survival times of series II in which $P<0.05$), but not quite significant in series I, in which the mice were on the high fat, high protein diet and the difference in the dietary level of phosphate between the test and control animals was less marked than in the other series.

*Discussion.* It should be pointed out that the present study concerns the acute or subacute toxicity of $P^{32}$. Any factor modifying the survival time figures in these experiments would act primarily by causing an increase or a decrease in the actual damage of the tissues, especially the hematopoietic tissues. This is probably the mechanism through which the enrichment of the diets with phosphate might cause a certain degree of protection. On the other hand, factors (such as the fatty infiltration of the liver or its partial damage) may probably impair or favor, directly or indirectly, the recovery of the damaged tissues. The action of such factors may not be detectable in experiments of acute toxicity, while it may become apparent in a study of the chronic effects of radiation, in which smaller doses of radioactivity would be used and the animals kept under observation for longer periods.

Our finding of an increased toxicity of $P^{32}$ in mice maintained on high protein diets is in apparent discrepancy with the very recent results of Jennings(6), who described a greater susceptibility to external radiation in protein depleted rats. These animals had been on a diet with a protein content (1.9%) much lower than that of our "low protein" diets (10%), and were irradiated after more than 2 months on this diet, when they had lost 25% of their initial weight. On the other hand, our mice were fed the diets for a much shorter period, and even after the injection of $P^{32}$, little or no loss of body weight occurred. It seems therefore that an increased radiosensitivity becomes apparent only in a condition of severe protein depletion, whereas, a moderate decrease in the dietary protein to a low maintenance level would cause a decrease rather than an increase in the susceptibility toward radiation injury. In this respect, our results may also be compared with others in the literature, showing that mice fed thyroid(7), or kept at low temperatures (a condition accompanied by an increased oxygen consumption)(8), become more susceptible to external radiation. One might postulate that the acute or subacute effects of radiation parallel the intensity of the metabolism and that the greater toxicity of $P^{32}$ in our mice on the high fat, high protein diet is, at least in part, related to the specific dynamic action of the dietary components (especially proteins).

*Summary.* In mice on a 10% casein, 32% fat diet, the $LD_{50}$ dose at the 21st day lies between 4 and 6 microcuries per g of body weight. Neither the fatty infiltration of the liver (induced by a deficiency of lipotropic factors, with or without partial poisoning with $CCl_4$), or its prevention (by supplementing the diet with choline) changes the survival figures. The time of 50% deaths, the percentage of survivors at the 21st day and the average survival time of mice injected with $P^{32}$ are highest with a diet low in fat and in protein, and are significantly decreased when the level of the fat, or of the protein, or both, are increased in the diet. Enrichment of the diets with inorganic phosphate seems to afford a moderate, but significant degree of protection against the injurious action of $P^{32}$.

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# Failure of Folic Acid to Influence Lethal Radiation Illness in Swine.* (17724)

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Following the synthesis of folic acid by Angier *et al.*(1), there were many reports on its beneficial effects in the therapy of pernicious anemia, sprue, nutritional macrocytic anemia and nutritional granulopenias of animals(2,3,4). Watson *et al.*(5), reported that folic acid might be of value in the therapy of refractory anemias and leukopenias, particularly the leukopenia following radiation therapy. This suggested that folic acid might be of value in the therapy of the pancytopenia that follows total body exposure to ionizing radiation. An experiment to investigate the effect of folic acid on the blood picture and lethality of total body exposure to x-ray was performed.

*Methods.* Eight adult swine(200 ± 50 lb) were given 400 r total body exposure to 1000 kv x-rays according to the method previously described(6). Folic acid was administered intramuscularly in doses of 45 mg per day to 4 of the 8 swine beginning the day after exposure and continuing until the death of the animal. The clinical course, the blood picture, sternal marrow, and post mortem pathological findings were recorded.

*Results.* The mortality was 100% in both the control untreated swine and in the folic-acid-treated swine. The average survival time of the treated swine was 14.7 days as compared with 14.2 days for the control untreated swine. The treated swine died between the 13th and 17th days and the untreated swine between the 12th and 16th days after exposure. The clinical course of both groups was similar. Scattered petechiae were visible on the second and third days after exposure. Hemorrhagic phenomena were prominent from the 10th day after exposure until death in all animals. One of the treated animals developed an abscess at the point of injection which was incised on the third day after exposure. Cultures were positive for *Hemophilus hemolyticus* and a diphtheroid. Although the pig died on the 13th day after exposure the abscess appeared to be healing despite the low white count.

In Table I are given the average group hematological values for both the control untreated and the treated swine. There is no significant difference between the two groups in respect to the differential cell count, or the various erythrocytic indices and mean values.

The gross and microscopic lesions in the control and test swine were similar in kind, distribution and in severity. It was not possible to distinguish by pathological examination between the treated and the untreated swine.

*Comments.* The mechanism by which folic acid acts in the therapy of macrocytic anemias is not clear. The role of folic acid in normal hematopoiesis is not well understood(4,7,8). How folic acid might ameliorate the severity of total body radiation illness would be difficult to understand in the light of our present knowledge. The reports of Watson *et al.*(5), and Davis(9) on the beneficial effect of folic

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* The Folvite solution (sodium salt of folic acid) was supplied by courtesy of Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.

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TABLE I.
Group Average Hematological Observations of Folic-Acid-Treated and Control Swine After Exposure to 400 r Total Body Irradiation

| Hematological observations of treated and untreated swine | | Control value | 1 | 2 | 3 | 4 | 6 | 7 | 10 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total WBC $\times 10^3/mm^3$ | Treated | 18.6 | 5.5 | 6.5 | 6.9 | 5.7 | 1.1 | 1.5 | 1.0 | 1.1 | 1.4 | 1.6 |
| | Untreated | 16.1 | 5.2 | 5.4 | 6.3 | 4.1 | 1.9 | 1.9 | 1.6 | 0.8 | 1.2 | |
| Total lymphocytes $\times 10^3/mm^3$ | Treated | 10.8 | 1.3 | 1.1 | 1.4 | 1.5 | 0.7 | 0.9 | 0.9 | 0.9 | 1.3 | 1.5 |
| | Untreated | 11.7 | 1.7 | 1.6 | 1.1 | 1.6 | 1.4 | 0.8 | 0.7 | 0.8 | | |
| Total neutrophiles $\times 10^3/mm^3$ | Treated | 7.6 | 4.1 | 5.3 | 5.5 | 4.1 | 0.4 | 0.6 | 0.1 | 0.2 | 0.1 | 0.1 |
| | Untreated | 5.3 | 3.4 | 3.7 | 5.1 | 2.5 | 0.5 | 1.1 | 0.9 | 0.1 | | |
| Total RBC $\times 10^6/mm^3$ | Treated | 6.7 | | | 6.6 | | 6.0 | 5.5 | | 3.3 | | 1.6 |
| | Untreated | 7.3 | | | 6.3 | | 6.4 | 5.9 | | 4.1 | | |
| Hemoglobin in g/100 cc | Treated | 13.8 | | | 13.9 | | 12.4 | 11.1 | | 6.3 | | |
| | Untreated | 13.8 | | | 14.0 | | 13.3 | 13.2 | | 8.5 | | |
| Hematocrit in % | Treated | 42. | | | 42. | | 38. | 35. | 35. | 18. | 13. | 9. |
| | Untreated | 44. | | | 39. | | 39. | 38. | 38. | 26. | 12. | |
| Reticulocytes in % | Treated | 2.0 | | | 0.3 | | | 0.2 | 0.9 | 1.4 | 1.2 | 0.7 |
| | Untreated | 2.0 | | | 0.3 | | | 0.3 | 0.2 | 0.3 | 3.2 | |
| Platelets $\times 10^3$ per $mm^3$ | Treated | 160. | | | 102. | | | 45. | 45. | 15. | | |
| | Untreated | 130. | | | 112. | | | 68. | 23. | 10. | | |
| Mean corpuscular volume in $\mu^3$ | Treated | 64. | | | 60. | | 63. | | 64. | 56. | | 56. |
| | Untreated | 60. | | | 62. | | 61. | | 65. | 65. | | |
| Mean corpuscular hemoglobin, $\mu\mu g$ | Treated | 21. | | | 21. | | 21. | | 20. | 19. | | |
| | Untreated | 19. | | | 22. | | 21. | | 22. | 21. | | |
| Mean corpuscular hemoglobin conc. in % | Treated | 31. | | | 30. | | 30. | | 31. | 29. | | |
| | Untreated | 32. | | | 28. | | 29. | | 29. | 30. | | |

acid in fractional therapeutic irradiation cannot be compared with single dose total body radiation as described in the present paper or in the recent report of Adams(10) where folic acid was shown to be ineffective in the treatment of irradiation leukopenia in the cat. Goldfeder *et al.*(11) present data showing that there is no appreciable effect on the hematopoietic system of mice given pyridoxine or folic acid, but that there is a significant prolongation of the survival time of irradiated mice receiving pyridoxine or folic acid. This later work is of great interest and should be confirmed on mice and other animals.

In the present study only 400 r of X-irradiation was used. This dose corresponds to approximately $LD_{85\text{-}30}$ for swine(6). It is possible that folic acid might be of benefit at other lethal doses. This possibility was not explored.

*Summary and conclusions.* 1. Eight swine were exposed to 400 r total body radiation. Four were treated with intramuscular injections of 45 mg of folic acid per day. The clinical and hematological courses were observed and postmortem examinations were performed. No differences were observed in the responses of the treated and untreated swine.

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# BAL and Testosterone Propionate in the Treatment of Mercuric Chloride Poisoning.* (17725)

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Acute toxic nephrosis still represents a therapeutic problem despite recent advances in therapy. BAL (2,3 dimercaptopropanol) has been shown to be a specific antidote for cases of this syndrome due to mercuric chloride. However, because of its own toxicity it will afford protection against only a certain lethal dose of the poison; experimental evidence indicates this is only a little less than twice the dose that is fatal for untreated animals(1). Previous studies have claimed that testosterone propionate by virtue of its renotrophic effect exerts a protective action when administered before(2) or shortly after (3) a given dose of mercuric chloride. It has also been claimed recently that testosterone may be effective in the treatment of the non-specific tubular damage of Asiatic cholera(4). In the investigation to be reported here the therapeutic effectiveness of testosterone in the acute toxic nephrosis of mercuric chloride poisoning was re-evaluated, and its use in conjunction with BAL was studied to determine if its renotrophic action had practical application in the treatment of tubular necrosis. The latter would be expected to be reflected by an increased survival rate of $HgCl_2$ poisoned animals treated with BAL and testosterone as compared to animals treated with BAL alone.

*Methods.* Mercuric chloride was dissolved in sterile distilled water to a concentration of 3 mg per cc. A selected dose was injected subcutaneously into the test animals. BAL and testosterone propionate† were obtained in the commercially available forms, being diluted when necessary to appropriate concentrations with corn oil. To avoid the error of using animals of different strains, rats of the "Sprague-Dawley" strain were employed. These animals were all males, weighing between 100 and 250 g. Water and food was always freely available to them. The kidneys of representative animals were studied histologically. Animals which were to be treated and the control animals were injected with mercuric chloride at the same time in each group of experiments. BAL and testosterone, or both, were given according to the schedule shown in Table I. Standard methods of statistical analysis were applied to the results and the conclusions drawn are based on these analyses. A preliminary survey indicated that 6 mg per kilo was approximately $LD_{50}$ for untreated animals. This dose of mercuric chloride was used throughout the study so that any therapeutic effect of testosterone would be more easily detectable. The dose of BAL chosen was the maximal tolerated effective dose(1). Testosterone was given over a wide range corresponding respectively to the following: (1) the dose used by Henderson *et al.*(4) and reported of value in the renal failure of Asiatic cholera, (2) the dose which is equivalent to the maximum clinically applicable in man, (3) the dose used by Longley in rats and reported to be effective in mercury poisoning, (4) the maximal dose considered feasible in rats of the size used. Two groups of unpoisoned animals given the largest

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† BAL, 10% solution in sesame oil (H. W. and D.), testosterone propionate in oil 50 mg/cc (Perandren), courtesy of Ciba Pharmaceutical Products, Inc.

TABLE I.
Dose Schedule of Treatment.

| Drug | Time and dose given, mg | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ½ | 6 | 24 | 48 hr | 3 | 4 | 5 | 6 | 7 | 8 days |
| BAL, per K | 30 | 15 | 15 | 15 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 |
| Testosterone | 50 | | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| ” | 10 | | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| ” | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| ” | .1 | | .1 | .1 | .1 | .1 | .1 | .1 | .1 | .1 |

TABLE II.
Effect of Treatment with BAL and Testosterone in Animals Treated with 6 mg/K Mercuric Chloride.

| Treatment | No. animals | No. dead | % mortality | $\chi^2$ | P |
|---|---|---|---|---|---|
| A. None | 27 | 15 | 56 | | |
| B. BAL | 16 | 3 | 19 | 5.59 | .02 |
| C. Testosterone | | | | | |
| (1) 0.1 mg/day | 10 | 4 | 40 | .71 | .42 |
| (2) 1 ” | 10 | 4 | 40 | .71 | .42 |
| (3) 10 ” | 10 | 4 | 40 | .71 | .42 |
| (4) 25 ” | 27 | 20 | 74 | 2.03* | .16 |
| D. BAL + testosterone | | | | | |
| (1) 0.1 mg/day | 10 | 1 | 10 | | |
| (2) 1 ” | 10 | 0 | 0 | | |
| (3) 10 ” | 10 | 1 | 10 | | |
| (4) 25 ” | 27 | 4 | 15 | | |

* Fatality rate in this group is *higher* than controls, but not significantly so.

doses of testosterone and the corn oil diluent were not clinically affected and gained weight normally.

*Results.* The effects of treatment with BAL and testosterone are shown in Table II. It is evident that BAL in the dose level used significantly reduced the mortality rate but was not completely protective. Testosterone, on the other hand, was totally ineffective in improving the survival rate.

In the second series of experiments (Table II) the effect of combined treatment with testosterone and BAL was compared to BAL alone to determine whether any synergistic action might be present. It is clear by inspection that the results were not appreciably improved. P values for each experiment were determined and were found not to be significant.

Histologic sections of kidneys from representative animals were studied by Dr. Simon Koletsky[‡] whose report follows:

"Microscopic study was made of kidneys from rats sacrificed 3, 6 and 9 days after injection of an $LD_{50}$ dose of mercuric chloride. Some animals received either BAL or testosterone, or both, while others were not treated and served as controls.

"Untreated animals and those treated with testosterone could not be separated by examining the sections, both showing the tubular necrosis typical of mercuric chloride poisoning. The sections showed that BAL did not prevent tubular necrosis; moreover, it was not possible to make an objective distinction in the amount of necrosis between BAL treated and untreated rats. No significant difference was noted in the rapidity and intensity of repair in recovery animals, whether treated or untreated. The beneficial effect of BAL may be chemical or physiological or explained on the basis of a quantitative alteration in the necrotic effect of mercury not detectable microscopically in these particular experiments."

*Discussion.* That testosterone stimulates renal tubular epithelium is well established, but the therapeutic significance of this fact remains unsettled. It would seem reasonable that a stimulating action upon the renal tubules could offer protection if the hormone were given before the renal insult, and Selye's work(2) supports this hypothesis. But whether such a stimulus is effective after tubular injury has already occurred is problematical. Although Longley's study appeared to favor such a possibility, the animals he used were not of uniform age and strain(5).

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[‡] Department of Pathology, Western Reserve University.

Henderson *et. al.* have suggested that testosterone was of benefit in the uremia of Asiatic cholera, stating: "We realize full well that in such a limited number of cases mortality statistics are not strictly applicable; nevertheless it is our firm belief that testosterone propionate is beneficial in this form of uremia." The evidence presented does not entirely support such a point of view. In their paper these authors do not present data on comparable controls, there was concomitant therapy with parenteral fluids and a sulfonamide, and in the majority of cases there are no blood chemical findings listed. Furthermore, cholera has a different mortality in different epidemics(6). With parenteral fluid therapy alone a mortality as low as 8% may be anticipated(7), results equal to those obtained by Henderson *et al.* with testosterone therapy. Regarding the renal pathology in this disease it has been stated that "in many instances there is little or no pathologic change, suggesting that kidney failure is largely extrarenal in origin"(8).

BAL, on the other hand, is definitely protective, as indicated by a significant reduction in mortality rate. It was rather surprising not to be able to determine the nature of this beneficial effect on microscopic study of the kidneys of the poisoned animals. Significant tubular necrosis was found in the BAL treated animals who would be expected to go on to recovery. One can only postulate that either the necrosis was quantitatively less severe than in the control animals, although this was not satisfactorily demonstrated in sections, or that the BAL protected enzyme systems in a way not reflected in tissue sections. The former possibility would be susceptible to proof by studying the kidneys of an adequate number of animals sacrificed shortly after poisoning and treatment.

*Summary.* 1. Male albino rats weighing between 100 and 250 g were poisoned with mercuric chloride and treated with BAL, testosterone propionate or a combination of both.

2. It was demonstrated that in rats as in other species BAL effectively combats the toxicity of mercuric chloride.

3. Testosterone propionate over a wide range of doses administered after mercuric chloride had no apparent effect upon the toxicity, either alone or in combination with BAL.

4. Histologic study of the kidneys failed to demonstrate any stimulating effect of testosterone on the damaged tubules. The definite protection afforded by BAL could not be correlated with any detectable microscopic changes in the tubules.

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# Effects of Folic Acid on Aminopterin Toxicity in the Immature Mouse. (17726)

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It is now well established that by suitable structural alterations synthetic vitamin analogues can be prepared which are inhibitory or "toxic" to the organism. In most cases the inhibition can be alleviated by adequate amounts of the corresponding vitamin, thus suggesting that the inhibition is of the competitive variety. With 4-amino-pteroylglutamic acid (aminopterin), however, an absolute deficiency of folic acid can be produced(1) which fails to respond even to massive doses of this vitamin. Franklin *et al.*(2) found that mice died within one week when fed purified rations containing 1 mg of 4-amino-pteroylglutamic acid per kg of diet. Folic acid failed to counteract this effect even when administered in doses as high as 100 mg per kg of diet, *i.e.* at a metabolite to inhibitor ratio of 100:1. Similar results have been observed in other species. Goldsmith *et al.*(3) demonstrated that the injection of estradiol into newly metamorphosed female frogs is followed within 2 to 3 weeks by marked enlargement and coiling of the oviducts. This effect may be largely counteracted by the administration of 4-amino-pteroylglutamic acid. As in the case of mice, however, doses of folic acid 100 times as large as the inhibitor failed to modify the aminopterin effect. Similar findings have been reported for the chick and rat. Karnofsky *et al.*(4) found that 4-amino-pteroylglutamic acid profoundly stunted the growth of chick embryos and produced a number of developmental abnormalities. Doses of folic acid 4000 times as large as the inhibitor failed to counteract these effects. In the rat toxic doses of 4-amino-pteroylglutamic acid incited within 6 days the occurrence of anorexia, diarrhea, atony of the entire gastrointestinal tract, adrenal hyperplasia, and atrophy of the thymus, spleen and bone marrow, with resulting leukopenia. Folic acid did not prevent these effects even when administered at a metabolite to inhibitor ratio of 600:1, although it did prolong the period during which animals remained symptom free(5). On the other hand Oleson *et al.*(6) reported that folic acid in doses approximately 25 times greater than the inhibitor counteracted the growth-retarding effect of 4-amino-pteroylglutamic acid in the chick and rat; and Hertz and Tullner(7) observed that folic acid in doses 300 to 400 times greater than the inhibitor counteracted the effects of 4-amino-pteroylglutamic acid in inhibiting the estrogen induced growth in the female genital tract of the stilbestrol-treated chick and the estradiol-treated ovariectomized rat. In the present communication data are presented indicating that folic acid in sufficiently high dosage will also counteract the deleterious effects of 4-amino-pteroylglutamic acid in the immature male mouse.

*Procedure.* The basal ration employed in the present experiment consisted of dextrin* 58, casein† 25, cottonseed oil (Wesson) 10,

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† Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

‡ Sure's Salt Mixture No. 1(8)

§ Ruffex, Fisher Scientific Co., St. Louis, Mo.

TABLE I.
Effects of Folic Acid, B Vitamins, and Liver on the Growth and Length of Survival of Immature Mice Fed Toxic Doses of Aminopterin for 20 days.*

| Dietary supplement | Avg initial body wt, g | % surviving* | Survival of decedents,† days | Gain in body wt in 20 days,† g |
|---|---|---|---|---|
| None | 13.8 | 100 | — | 5.1 ± 1.1<br>(12) |
| ” | 14.7 | 0 | 8.4 ± .6 | — |
| B complex vit. mixture (without vit. $B_{12}$) | 15.6 | 0 | 6.8 ± .3 | — |
| Vit. $B_{12}$ | 12.2 | 0 | 7.3 ± .5 | — |
| Whole liver | 14.6 | 16.7 | 8.4 ± .7 | 0.5 ± 0.1<br>(2) |
| Liver conc. 1-20 | 14.9 | 0 | 8.2 ± .7 | — |
| ” residue | 15.0 | 0 | 8.7 ± .5 | — |
| Folic acid (90 mg/kg of diet) | 14.1 | 25 | 12.9 ± .3 | 0.1 ± 0.9<br>(3) |
| ” ” (490 ” ” ”) | 14.6 | 100 | — | 8.2 ± 1.0<br>(12) |

The values in parentheses indicate the No. of surviving animals on which the averages are based.

* Data are based on the top 12 of the 15 mice constituting each group. In all of the dietary regimens, except the first in which no aminopterin was fed, the dosage of aminopterin was 1 mg per kg of diet.

† Including standard error of the mean calculated as follows $\sqrt{\frac{\epsilon d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

salt mixture[‡] 5, and cellulose[§] 2%. To each kg of the above diet were added the following synthetic vitamins: thiamine hydrochloride 20 mg, riboflavin 20 mg, pyridoxine hydrochloride 20 mg, nicotinic acid 60 mg, calcium pantothenate 60 mg, biotin 4 mg, folic acid 10 mg, p-aminobenzoic acid 400 mg, inositol 800 mg, 2-methyl-naphthoquinone 10 mg, and choline chloride 2 g. Each mouse also received once weekly a vitamin A-D concentrate,[||] containing 25 U.S.P. units of vitamin A and 2.5 U.S.P. units of vitamin D, and 1.5 mg of alpha-tocopherol acetate. For the tests 135 male mice of the CC-1 strain[¶] were selected at the age of 24 to 27 days and an average weight of 14.6 g. They were placed in metal cages with raised screen bottoms to prevent access to feces and were fed the following diets *ad libitum*: (a) basal ration alone (b) basal ration plus aminopterin. In addition to the basal ration plus aminopterin, diet (c) contained per kg twice as much thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, nicotinic acid, calcium pantothenate, biotin, folic acid, p-aminobenzoic acid, inositol and 2-methyl-naphthoquinone as was present in the basal ration; diet (d) contained 30 $\gamma$ of vitamin $B_{12}$[**] per kg; diet (e) 10% of whole liver power;[††] diet (f) 2.5% of liver concentrate 1:20;[‡‡] diet (g) 10% of liver residue;[§§] diet (h) 90 mg of folic acid per kg; and diet (i) 490 mg of folic acid per kg. Aminopterin (4-aminopteroylglutamic acid)[|||] was incorporated in the above diets at a level of 1 mg per kg of diet. The supplements replaced an equal

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[||] Napco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per g.

[¶] Animals were obtained from a local breeder. The CC-1 strain was derived from crossbreeding the Webster and ABC strain.

[**] Cobione (Crystalline Vitamin $B_{12}$ Merck), Merck and Co., Rahway, N.J.

[††] Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

[‡‡] Liver Concentrate Powder 1-20, Wilson Laboratories, Chicago, Ill.

[§§] Extracted Liver Residue, Wilson Laboratories, Chicago, Ill.

[|||] We are indebted to Dr. E. L. R. Stokstad of Lederle Laboratories, Pearl River, N.Y., for providing the aminopterin employed in the present experiment.

amount of dextrin. Feeding of the 15 mice in each group was continued for 20 days or until death, whichever occurred sooner.

*Results.* The findings are summarized in Table I. To minimize variations in averages due to early deaths, infection and atypical responses on the part of individual mice, the data were computed on the basis of the top 12 animals in each group. In agreement with earlier findings(2) the mice lost weight and failed to survive when fed a purified ration containing 1 mg of aminopterin per kg of diet. The supplements of whole liver powder, water-soluble liver extract and water-insoluble liver residue were without significant effect. Doubling the B vitamin content of the experimental diet and adding vitamin $B_{12}$ also proved ineffective. Only folic acid of all of the substances tested exerted a protective effect. A diet containing 100 mg of folic acid per kg of diet prolonged slightly the average survival time of the mice. Folic acid at a level of 500 mg per kg of diet, however, completely counteracted the deleterious effects of aminopterin on growth and survival under conditions of the present experiment.

*Summary.* Immature male mice lost weight and failed to survive when fed a purified ration containing 1 mg of aminopterin per kg of diet. These effects were completely counteracted by folic acid during an experimental period of 20 days when administered at a level of 500 mg per kg of diet.

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## Antimicrobial Action of Terramycin *in Vitro* and *in Vivo*. (17727)

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In a recent communication(1), the isolation of a new antibiotic, terramycin, has been reported. Terramycin is a highly active antimicrobial agent which is effective both *in vitro* and *in vivo* against certain of the pathogenic bacteria and certain of the rickettsiae (1,2). The studies reported herein were carried out in an attempt to evaluate more extensively the antimicrobial action of terramycin *in vitro* and its chemotherapeutic action in acute experimental infections in animals.

*In vitro activity. 1. Materials and methods.* Partially purified and crystalline terramycin,* terramycin hydrochloride, terramycin sulfate, and 2 forms of the sodium salt of terramycin (pH 8.5 and 9.8)† have been used to date in determining the sensitivity of various microorganisms to terramycin. Aureomycin

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*All of the terramycin used throughout this study was prepared by the Departments of Biochemical and Chemical Research and Development, Chas. Pfizer & Co., Inc. We are indebted especially to Dr. B. A. Sobin and Dr. P. P. Regna for the preparation of large quantities of the antibiotic for this study during the early stages of its development, and to Mr. J. H. Kane and Dr. R. A. Patelski for their advice and assistance throughout the study.

† The pH of the sodium salt of terramycin in dilute aqueous solution is approximately 9.8. It was observed early in the study of terramycin that dilute solutions of its sodium salt may be adjusted to pH 8.5 without precipitation of the antibiotic. It is recognized that such solutions probably consist of mixtures of terramycin and its sodium salt. Although this form cannot be prepared readily as a solid product, its pH and its satisfactory solubility have made it a more desirable preparation for certain preliminary laboratory studies than terramycin hydrochloride (pH 1.5), terramycin sulfate (pH 1.5), or the true sodium salt of terramycin (pH 9.8).

hydrochloride‡ and terramycin hydrochloride were tested simultaneously against a limited series of these microorganisms, in order to obtain a base line for interpretation of results obtained by the method used.

In the interests of convenience, the unit of terramycin has been defined as one microgram of the pure amphoteric compound. The activities of the salts of terramycin are stated in terms of the equivalent weight of pure terramycin(3). The antimicrobial activity of terramycin *in vitro* has been evaluated by standard methods used for other antibiotics. The method used in this laboratory for determination of the sensitivity of microorganisms to terramycin is a modification of that previously described by Lenert and Hobby (4) for streptomycin.

In all instances, the antimicrobial agent was dissolved in broth in a concentration of 100 μg per ml and the resultant solution passed through a Seitz filter to ensure sterility.§ Two-fold serial dilutions of this solution of terramycin were made in nutrient broth, and, in the case of bacteria, 0.9 ml of each solution was seeded with 0.1 ml of a $10^{-1}$ or $10^{-4}$ dilution of a standardized broth or blood broth culture of the organism under test. Except when otherwise specified, 16 to 18 hour broth cultures were used throughout. These cultures were diluted in broth to a constant density immediately prior to use. A density allowing 75 to 85% transmission on a Photovolt Lumetron No. 400 was chosen as standard. In the case of rapidly growing organisms, the $10^{-4}$ dilution was used. In the case of poorly growing organisms, a $10^{-1}$ dilution was more satisfactory. Incubation was carried out at 37°C for a period of 48 hours. The sensitivity of an organism was accepted as the least amount of antibiotic capable of causing complete inhibition of growth as evidenced by absence of gross turbidity. In view of the two-fold nature of the serial dilution assay used, however, the sensitivity may have been somewhat greater than that indicated by the accepted end points.

Levinthal's broth was used for studies on *H. influenzae*(5) and the Bordet-Gengou medium for those on *H. pertussis;* an incubation period of 72 hours was used for *H. pertussis*. All studies on *M. tuberculosis* were carried out in the Dubos liquid medium containing Tween 80, as described in a previous communication(6). An incubation period of 7 days at 37°C proved satisfactory for this purpose.

In the case of fungi, Sabaraud's agar was used routinely(7). High concentrations of terramycin were obtained by dilution and Seitz filtration in Sabaraud's broth, followed by subsequent mixing with an equal volume of Sabaraud's agar containing twice the normal concentration of agar. Sabaraud's agar slants (10 ml) containing varying concentrations of terramycin then were inoculated with 0.1 ml of a heavy suspension of a 9-day culture of the organism under test. All tests were incubated at 37°C and the amount of growth read at 3 and 5 days. The sensitivity of an organism in this case was accepted as the least amount of antibiotic necessary to inhibit growth as evidenced by absence of colonial growth on the surface of the agar slant. A comparison of the *in vitro* activity of terramycin and its salts has indicated that there is no significant difference in their

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‡ We are indebted to Dr. Stanton M. Hardy of the American Cyanamid Company for the aureomycin hydrochloride used in these studies.

3. Kersey, R. C., *J. Am. Pharm. Assn., Scient. Edition*, 1950, in press.

4. Lenert, T. F., and Hobby, G. L., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 235.

§ Studies on the degree of adsorption of terramycin onto Seitz filter pads have indicated that little or no terramycin is removed from solution during filtration of small volumes (20 to 50 ml) of broth or serum broth solutions of terramycin (50 to 100 μg per ml). Fifty to 75% of terramycin present in aqueous solutions, in contrast, may be removed from solution during filtration. Throughout the studies reported herein, all terramycin, therefore, was dissolved in plain or 25% serum broth prior to filtration.

5. Alexander, H., *et al.*, *J. Exp. Med.*, 1947, v85, 329, 607.

6. Hobby, G. L., Lenert, T. F., and Dougherty, N., *Annals of the N. Y. Acad. of Sci.*, 1949, v52, 775.

7. Hobby, G. L., Regna, P. P., Dougherty, N., and Steig, W. E., *J. Clin. Invest.*, 1949, v28, 927.

TABLE I.
Antimicrobial Action of Terramycin *in vitro.*

I. Antibacterial Activity of terramycin

| Microörganisms | Sensitivity: μg of terramycin per cc—37°C 24 hr | 48 hr |
|---|---|---|
| *Strep. hemolyticus, Group A* | | |
| C203Mv | <.3 | 1.5 |
| Type 18 | .6 | 1.5 |
| Strain Br-Mn. | 1.5 | 1.5 |
| *Strep. hemolyticus*, Group C | | |
| Griffith 20 | 2.5 | 21. |
| ” 21 | .6 | 1.5 |
| ” 7 | .6 | .6 |
| H 76 | <.3 | <.3 |
| H 81 | .6 | .6 |
| *D. pneumoniae* | | |
| I/230, type I | <.02 | .02 |
| D/39, ” II | <.3 | <.3 |
| A66, ” III | <.3 | <.3 |
| M$_3$R | <.3 | 2.5 |
| BR. | <.3 | .5 |
| Sch. | <.4 | <.4 |
| *Strep. viridans* (Hawth.) | <.3 | 2.5 |
| Enterococcus | .6 | .6 |
| *Staph. aureus* (H) | .7 | 1.5 |
| (Br.) | 1.5 | 1.5 |
| *Staph. albus* (Bell) | 168. | >168. |
| *C. diphtheriae* (No. 210) | .6 | .6 |
| *Bc. subtilis* (W) | .4 | .7 |
| (PCI-224) | 42. | 42. |
| (No. 6633) | .6 | .6 |
| *Cl. welchii* | <.3 | 11. |
| *N. catarrhalis* (ATCC) | 2.5 | 2.5 |
| *N. meningitidis* | <.3 | .5 |

| Microörganisms | Sensitivity: μg of terramycin per cc—37°C 24 hr | 48 hr |
|---|---|---|
| *Bruc. bronchiseptica* | <.3 | 1.5 |
| *H. influenzae* (St.) | 2.0 | 4.0 |
| (DeR.) | 2.5 | 2.5 |
| *H. pertussis* | | 5.0‡ |
| *K. pneumoniae* (Kin.) | 1.5 | 2.5 |
| *K. pneumoniae* (Nol.) | .9 | 1.5 |
| Streptomycin-resistant mutant | 21. | 21.0* |
| *A. aerogenes* | 1.5 | 2.5 |
| Streptomycin-resistant mutant | 2.5 | 2.5* |
| Unidentified Gram Neg. Rod (SMR) | 5.0 | 5.0* |
| *E. coli* (BW 41) | 2.5 | 5.0 |
| (W 87) | 2.5 | 5.0 |
| (Bristol) | 5.0 | 5.0 |
| *Ps. pyocyaneus* (St.) | 5.0 | 21. |
| (ATCC 9027) | 21. | 21. |
| *Proteus vulgaris* | >167. | >167. |
| *S. typhosa* (St.) | 1.5 | 2.5 |
| Streptomycin-resistant mutant (NAII) | <.3 | 1.5* |
| Streptomycin-resistant mutant (NAIV) | .6 | 1.5* |
| *S. paratyphosa* (Fr.) | 2.5 | 5.0 |
| *S. dysenteriae* (Duf.) | 2.5 | 5.0 |
| *S. typhi murium* (L.I.) | 2.5 | 5.0 |
| *S. typhi murium* (B.I.) | 3.0 | 5.5 |
| *S. newport* (B.I.) | 5.5 | 5.5 |
| *S. enteritidis* (B.I.) | 3.0 | 5.5 |
| *S. cholera suis* (B.I.) | 3.0 | 5.5 |

* *K. pneumoniae* strain requires 1400 μg streptomycin per cc for inhibition; *A. aerogenes* strain requires 2800 μg; both strains of *S. typhosa* require >5000 μg; and the unidentified gram negative rod requires >100,000 μg of streptomycin for inhibition.

† Readings in this instance were made at 72 hr (37°C).

II. Tuberculostatic Action of Terramycin Hydrochloride:

| Microörganisms | Sensitivity: μg of terramycin per cc 5 days—37°C | 12 days—37°C | Sensitivity to streptomycin, μg per cc (12 days) |
|---|---|---|---|
| H$_{37}$Rv (No. 2678) | 3.0 | 13.0 | <.3 |
| Freshly isolated strains | | | |
| Ni. | 3.0 | 16.0 | 6.0 |
| Mu. | 1.5 | 26.0 | >1000. |
| Mo. | 3.0 | 13.0 | .5 |
| Be. | 3.0 | 26.0 | 25.0 |
| St. | 1.5 | 26.0 | 5000. |

antimicrobial action. Terramycin, in concentrations as high as 298 μg per ml of medium, was incapable of inhibiting the growth of nine strains of fungi (*Nocardia asteroides, Sporotrichum schenkii, Microsporum canis, Trichophyton rubrum, Cryptococcus neoformans, Trichophyton mentagrophytes, Monilia albicans, Epidermophyton floccosum,* and *Microsporum audouini*).

Preliminary observations on the nature of the *in vitro* activity of terramycin were made by means of growth curves(8). The rate of growth of *Streptococcus hemolyticus* was

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8. Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 281.

TABLE II.
Antimicrobial Action of Terramycin Hydrochloride: Comparison with Aureomycin Hydrochloride.

| | Sensitivity: μg (total wt) per cc | |
|---|---|---|
| | Terramycin HCl | Aureomycin HCl (A377) |
| *Strep. hemolyticus* (C203Mv) | .9 | .9 |
| *D. pneumoniae* (I/230) | .9 | .9 |
| *Staph. aureus* (H) | .9 | .9 |
| *K. pneumoniae* | 2.0 | 3.5 |
| *A. aerogenes* | 2.0 | 7.0 |
| *E. coli* (BW 41) | 7.0 | 14.0 |
| " (W 87) | 7.0 | 14.0 |
| *S. typhosa* | 7.0 | 7.0 |
| *S. dysenteriae* | 7.0 | 14.0 |
| *S. paratyphosa* | 7.0 | 14.0 |
| *Proteus vulgaris* | 450. | >450. |
| *Pseud. pyocyaneus* | 28. | 57. |
| *Brucella bronchiseptica* | .9 | .9 |

Tests carried out simultaneously under identical conditions.

Incubation period 48 hr at 37°C.

measured in the presence and absence of varying concentrations of terramycin, by determination of the number of organisms per ml at varying times during the incubation period.

2. *Results.* The results of these studies are illustrated in Tables I, II, and III, and in Fig. 1 and 2. From the data available, it appears that that terramycin is more like penicillin than streptomycin in that its action is influenced only slightly by environmental factors. As is the case with other antibiotics, however, the concentration of terramycin present influences markedly the rate and extent of inhibition of growth of a given organism. Terramycin is a highly bacteriostatic agent although, under certain conditions, bactericidal action may be apparent. It is effective *in vitro* in low concentrations against a wide variety of microorganisms belonging to both the gram-positive and gram-negative groups.

*In vivo activity. 1. Materials and methods.* The preparations of terramycin used in these experiments were comparable with those which have been described above. Procedures employed in the evaluation of other antimicrobial agents have proved highly satisfactory for study of the chemotherapeutic action of terramycin, and the methods used in this laboratory were ones which have been described in detail in a communication concerned with penicillin(9). With the exception of those experiments pertaining to the chemotherapeutic action of terramycin against infections due to *Borrelia recurrentis,* all tests were carried out in male white mice (Rockland Regular Strain) weighing 15 to 20 g. In the case of *S. typhosa, K. pneumoniae,* and *H. influenzae,* cultures were diluted in mucin prior to injection; in the case of *H. pertussis,* cultures were diluted in Casamino acid, while in other instances dilutions were made in nutrient broth. Fourteen to 16 hour broth or blood broth cultures of all organisms, except *H. influenzae* and *H. pertussis,* were used. In the case of *H. influenzae,* a suspension of organisms, washed from a 5½-hour Levinthal's agar plate and having a density equivalent to 75% transmission (Photovolt Lumetron No. 400) was used; in the case of *H. pertussis,* a suspension of organisms in 1% Casamino acid, made from a 48-hour Bordet-Gengou plate, and having a density equivalent to 35% transmission, was used. Mice were infected with *H. influenzae* by the intraperitoneal injection of 1 ml of a $10^{-5}$ dilution of the standardized suspension of culture, diluted in mucin. Mice were infected with *H. pertussis* by the intracerebral injection of 0.01 ml of the undiluted suspension of culture described above. Animals were infected with all other organisms by the intraperitoneal injection of 1.0 ml quantities of varying dilutions of the culture under test.

All mice were treated with terramycin either in a single dose administered one-half hour after infection, or in divided dosage, twice daily beginning one hour after infection and continuing for a period of 2½ days. Both the subcutaneous and oral routes of administration were used.

Rats belonging to the Sherman strain and weighing approximately 90 to 110 g were infected intraperitoneally with 0.6 ml of citrated hearts' blood from previously infected rats in which the presence of *Borrelia*

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9. Hobby, G. L., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 296; *J. Bact.*, 1947, v54, 305.

TABLE III.
Antimicrobial Action of Terramycin: Comparison of the Action of Terramycin and Its Salts.

| | Sensitivity: μg of terramycin per cc | | | |
|---|---|---|---|---|
| Organism | Terramycin | Terramycin hydrochloride | Terramycin sulfate | Terramycin sodium salt* |
| *Strep. hemolyticus* (C203Mv) | 5.0 | 3.0 | 3.0 | 3.5 |
| *D. pneumoniae* (I/230) | <1.5 | <1.5 | <1.5 | <1.5 |
| *Staph. aureus* (H) | <1.5 | <1.5 | <1.5 | <1.5 |
| *Bc. subtilis* (W) | <1.5 | <1.5 | <1.5 | <1.5 |
| *K. pneumoniae* | 5.0 | 6.0 | 3.0 | <1.5 |
| *A. aerogenes* | 5.0 | 6.0 | 5.5 | 3.5 |
| *E. coli* (BW 41) | 11.0 | 6.0 | 5.5 | 3.5 |
| *E. coli* (W 87) | 11.0 | 6.0 | 5.5 | 6.5 |
| *Ps. pyocyaneus* | 42.0 | 23.0 | 11.0 | 26.0 |
| *S. dysenteriae* | 21.0 | 12.0 | 5.5 | 6.5 |
| *S. typhosa* | 5.0 | 6.0 | 3.0 | 3.5 |
| *S. paratyphosa* | 11.0 | 12.0 | 5.5 | 26.0 |
| *Proteus vulgaris* | >600 | >742 | >716 | >837 |
| *Bruc. bronchiseptica* | 2.5 | 3.0 | <1.5 | <1.5 |
| *M. tuberculosis* | | | | |
| $H_{37}Rv$ Isolate No. 2678 | | 6.5 | 6.0 | 7.5 |
| Freshly isolated strains | | | | |
| St. | | 6.5 | 6.0 | 4.5 |
| *Be.* | | 6.5 | 6.0 | 7.5 |
| *Mo.* | | 6.5 | 6.0 | |
| *Mu.* | | 4.0 | 6.0 | 3.5 |
| *Ni.* | | 6.5 | 6.0 | 7.5 |

Except in the case of *M. tuberculosis*, incubation was carried out for 72 hours at 37°C. Cultures of *M. tuberculosis* were incubated for 7 days at 37°C. Longer incubation was not used in view of the fact that the stability of terramycin at 37°C (pH 7.0-7.8) does not permit accurate readings after longer periods of time.

* pH 8.5 sodium salt of terramycin used.

*recurrentis*[‖] was apparent by examination of Giemsa-stained blood smears. A portion of the infected rats were held as controls. Terramycin was administered to the remaining animals by the intramuscular route, once or twice daily, beginning 1 hour after infection and continuing for 4 days.

2. *Results.* The results of extensive studies, carried out by the methods described, to determine the chemotherapeutic action of terramycin against a variety of experimental infections in animals are illustrated in Tables IV and V. From these data, it is apparent that terramycin is a highly active antimicrobial agent capable of protecting mice against infections due to *Streptococcus hemolyticus*, *D. pneumoniae*, *K. pneumoniae*, *H. influenzae*, *H. pertussis*, *S. typhosa*, *S. newport*, *S. enteritidis* and *S. cholerae suis.*[¶] It is capable, furthermore, of suppressing temporarily experimental infections in mice due to *S. typhimurium.* It is effective when administered by the oral as well as the parenteral routes. Terramycin is ineffective in the control of experimental mouse infections due to *Ps. pyocyaneus* or *Proteus vulgaris.* Preliminary studies in rats infected with *Borrelia recurrentis* suggest that 0.82 mg of terramycin per 100 g of body weight, administered intramuscularly once daily for 4 days, is sufficient to sterilize the blood.

Data on the chemotherapeutic action of terramycin against experimental mouse infections due to *M. tuberculosis* will be reported in detail elsewhere(10). Preliminary studies indicate, however, that 7.1 mg of terramycin per day administered subcutane-

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[‖] We are indebted to Dr. Fordyce Heilman of the Mayo Clinic, Rochester, Minn., for the strain of *Borrelia recurrentis* used.

[¶] Studies on the chemotherapeutic action of terramycin against experimental infections due to the Clostridia are in progress and will be reported at a later date. The observations made so far would suggest that this antimicrobial agent is effective against this group of organisms.

10. Hobby, G. L., Lenert, T. F., Donikian, M., and Pikula, D., unpublished data.

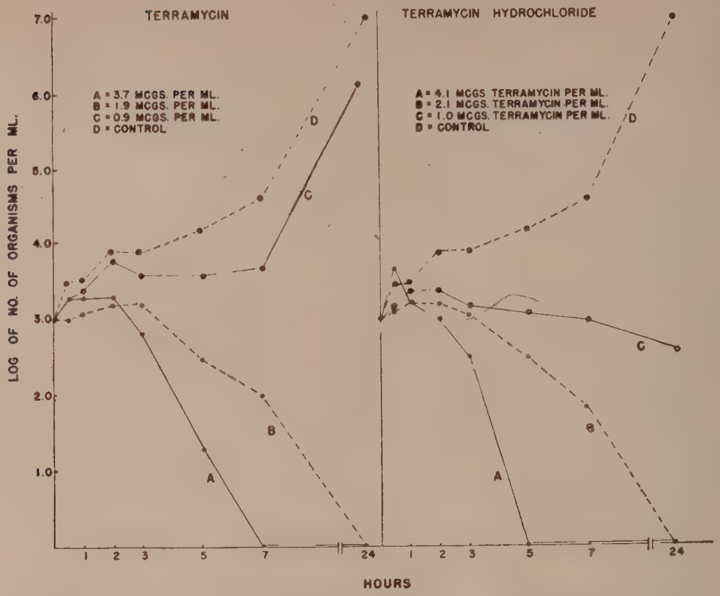

FIG. 1.

Comparison of the rate of action of varying concentrations of terramycin and terramycin hydrochloride against *Streptococcus hemolyticus* ($C_{203}$Mv) *in vitro.*

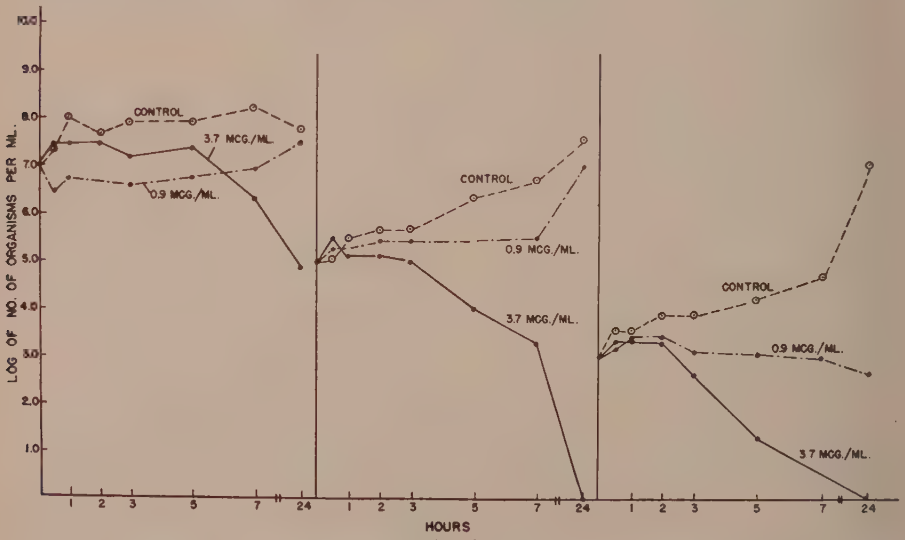

FIG. 2.

The rate action of terramycin against varying numbers of *Streptococcus hemolyticus* (C203Mv) *in vitro.*

ously is sufficient to exert a suppressive effect on the tuberculous infection in mice. Terramycin is absorbed rapidly into the circulatory system following oral or parenteral administration.

Significant concentrations may be detected in the urine of injected animals in a biologically active form(11). Terramycin is a substance of low toxicity. Preliminary studies in mice indicate that at

TABLE IV.
Chemotherapeutic Action of Terramycin and Its Salts Against Experimental Infections in Mice.

| | Terramycin | | | | | | |
|---|---|---|---|---|---|---|---|
| | Injection schedule | | | $CD_{50}$ (19/20 Confidence Limits): Mg per 20 g mouse) | | | |
| Microörganism | Frequency | Total No. | Route | Amphoteric | HCl | $SO_4$ | Sodium salt |
| *Strep. hemolyticus* (C203Mv) | b.i.d. | 5 | Subcu. | .03 ( .01- .07) | .01 ( .01- .07) | .04 ( .02- .07) | .03 ( .01- .06) |
| | q.d. | 1 | ,, | .10 ( .06- .16) | .35 ( .23- .54) | .35 ( .20- .63) | .25 ( .17- .36) |
| | b.i.d. | 5 | Oral | .36 ( .08- 1.62) | .74 ( .49- 1.11) | .69 ( .33- 1.45) | .50 ( .19- 1.30) |
| | q.d. | 1 | ,, | 2.5 ( 1.84- 3.40) | 1.20 ( .67- 2.16) | 3.10 (1.55- 6.20) | 2.50 (1.32- 4.75) |
| *D. pneumoniae* (I/230) | b.i.d. | 5 | Subcu. | .26 ( .16- .42) | .27 ( .20- .36) | .24 ( .18- .32) | .30 ( .19- .47) |
| | q.d. | 1 | ,, | 1.3 ( .72- 2.34) | 2.6 (1.62- 4.16) | 2.3 (1.05- 5.06) | 2.20 (1.58- 3.06) |
| | b.i.d. | 5 | Oral | 17 (12.69-22.78) | 6.40 (4.92- 8.30) | 8.10 (6.43-10.20) | 6.80 (5.20- 8.84) |
| | q.d. | 1 | ,, | >25 | 25 (16.45-38 ) | >25 | >25 |
| *K. pneumoniae* (Nol.) | b.i.d. | 5 | Subcu. | .18 ( .11- .30) | .098 ( .04- .22) | .19 ( .11- .33) | .10 ( .06- .16) |
| | q.d. | 1 | ,, | .68 ( .40- 1.16) | .13 ( .07- .23) | .31 ( .18- .54) | .50 ( .35- .72) |
| | b.i.d. | 5 | Oral | .84 ( .21- 3.36) | 2.3 (1.53- 3.45) | 2.50 (1.75- 3.58) | 1.1 ( .69- 1.76) |
| | q.d. | 1 | ,, | 2.40 ( 1.32- 4.37) | 1.60 ( .50- 5.14) | 4.50 (3.00- 6.75) | 3.20 (2.37- 4.30) |
| *S. typhosa* | b.i.d. | 5 | Subcu. | .08 ( .06- .11) | .24 ( .12- .48) | .16 ( .08- .31) | .34 ( .16- .71) |
| | q.d. | 1 | ,, | .56 ( .37- .84) | .60 ( .16- 2.88) | 1.00 ( .55- 1.82) | .96 ( .41- 2.26) |
| | b.i.d. | 5 | Oral | 12 ( 8.89-16.2 ) | 10.5 (6.18-17.85) | Approx. 33 | |
| | q.d. | 1 | ,, | 25 (13.51-46.25) | 7.0 (3.18-15.40) | 21 (7.5 -58.8 ) | 13 (9.29-18.2 ) |
| *H. influenzae* | b.i.d. | 5 | Subcu. | .12 ( .02- .66) | | | |
| | q.d. | 1 | ,, | 1.7 ( .36- 7.99) | | | |
| *H. pertussis* | b.i.d. | 5 | ,, | Approx. .7 | | | |
| | q.d. | 1 | ,, | 4.1 ( 2.28- 7.38) | | | |

In all instances, a minimum of 10 mice per dosage level and 4 to 8 dosages were used to determine each of values above. The $CD_{50}$ (19/20 Confidence Limits) values were calculated according to the method of Litchfield & Wilcoxon(13).

TABLE V.
Chemotherapeutic Action of Terramycin Against Experimental Infections in Mice.

| | Terramycin (Amphoteric) | | | | |
|---|---|---|---|---|---|
| | Injection schedule | | | CD$_{50}$ (19/20 Confidence Limits): mg per 20 g mouse Observation period | |
| Microörganisms | Frequency | Total No. | Route | 96 hrs | 10 days |
| *S. typhi murium* | b.i.d. | 5 | Subcu. | 0.13 ( .08- .22) | >1.00 |
| | b.i.d. | 5 | Oral | 3.0 (1.76-5.10) | 44 (18.33-105.6 ) |
| *S. cholerae suis* | b.i.d. | 5 | Subcu. | 0.10 ( .04- .26) | 0.22 ( 0.09- 0.53) |
| | b.i.d. | 5 | Oral | 2.0 (1.18-3.4 ) | >20 |
| *S. enteritidis* | b.i.d. | 5 | Subcu. | 0.09 ( .05- .17) | 3.8 ( 1.09- 13.3 ) |
| | b.i.d. | 5 | Oral | 6.2 (3.9 -9.9 ) | >20 |
| *S. newport* | b.i.d. | 5 | Subcu. | 0.05 ( .02- .06) | 0.18 ( 0.08- 0.4 ) |
| | b.i.d. | 5 | Oral | 2.0 ( .57-7. ) | 7.2 ( 5.14- 10.08) |

In all instances, min. of 10 mice per dosage, 4 to 8 dosages were used to determine each value above. The CD$_{50}$ (19/20 Confidence Limits) values were calculated according to the method of Litchfield & Wilcoxon(13).

TABLE VI.
Subacute Toxicity of Terramycin in Mice.

| Dosage (mg/20 g mouse) | No. doses per day | No. of days | Route of administration | No. of mice | % survival |
|---|---|---|---|---|---|
| 7.45 | 2 | 5 | Oral | 10 | 100 |
| 3.72 | 2 | 5 | ,, | 10 | 100 |
| 1.86 | 2 | 5 | ,, | 10 | 100 |
| 11.18 | 2 | 5 | Subcutaneous | 10 | 60 |
| 7.45 | 2 | 5 | ,, | 10 | 90 |
| 3.72 | 2 | 5 | ,, | 10 | 80 |
| 11.18 | 1 | 5 | ,, | 10 | 60 |
| 7.45 | 1 | 5 | ,, | 10 | 80 |
| 3.72 | 1 | 5 | ,, | 10 | 100 |

least 375 mg may be administered orally twice daily for at least 5 days with no untoward symptoms. By the subcutaneous route, 185 mg per kg body weight may be administered once daily without toxicity. (Table VI). Extension studies by P'an and his associates(12) in mice and other species of animals further illustrate the low toxicity of this compound.

*Discussion and summary.* The data reported in this and in a previous communication by Finlay *et al.*(1) emphasize the strong antimicrobial activity of terramycin and its salts against a wide variety of aerobic and anaerobic gram-positive and gram-negative bacteria, and against certain of the rickettsiae. In addition, attention is called to the possible tuberculostatic activity of this compound. Terramycin is absorbed readily following oral or parenteral administration and is a highly effective chemotherapeutic agent, active against a variety of experimental infections in mice. A comparative study of the chemotherapeutic activities of terramycin and its salts indicates that the highly insoluble amphoteric form of terramycin as well as its more soluble salts are all chemotherapeutically effective. In most instances, the CD$_{50}$ of the hydrochloride has been less than that of the other forms of terramycin, especially when administered by the oral route. More satisfactory results, as indicated by lower CD$_{50}$ values, have been obtained in some in-

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11. Hobby, G. L., Reed, W., Rinne, D., Powers, M., and D'Ambrosia, A., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 511.

12. P'an, S. Y., *et al.*, *J. Pharm. and Exp. Therap.*, 1950, in press.

13. Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharm. and Exp. Therap.*, 1949, v95, 99.

fections, however, with terramycin (amphoteric) when therapy was carried out by means of a single injection. It is possible that this enhanced chemotherapeutic effect, observed in these infections when treatment is carried out by a single injection only, may be a reflection of the low solubility and slow absorption of terramycin in the amphoteric form.

The toxicity of terramycin has been studied extensively by P'an and his associates(12). It is possible that the main virtue of terramycin resides in its remarkably low toxicity and low chemotherapeutic index. Terramycin, however, is a highly effective chemotherapeutic agent against experimental animal infections and it is believed that terramycin may prove of value in the treatment of certain human infections due to terramycin-sensitive organisms. A therapeutic evaluation of terramycin in human infections, therefore, has been initiated.

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## Absorption and Excretion of Terramycin in Animals. (17728)

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The antimicrobial activity of terramycin has been described in detail(1). The present study was undertaken in order to determine the rate of absorption of terramycin in experimental animals and to evaluate the differences, if any, in the rates of absorption of the various salts of terramycin.

*Materials and Methods.* Throughout this study, partially purified and crystalline terramycin, terramycin hydrochloride, terramycin sulfate, and 2 forms of the sodium salt of terramycin* prepared from crystalline terramycin have been used. Absorption and excretion studies have been carried out in rabbits and dogs. Terramycin has been administered in aqueous solution or suspension by the intravenous, intramuscular, or oral route. In all instances specimens of sera were separated from the blood clot on the day of bleeding and all serum and urine samples frozen on the day of collection. Samples were thawed once only at the time of test. The concentrations of terramycin in serum and urine may be determined by the standard methods used for penicillin. In this laboratory a modification of the method of Tompsett and his associates(2) has been used throughout. The procedure differs from those of Rammelkamp, Kirby, and other investigators in that the increments are small, the amount of serum in each tube is constant and the concentration of organisms is small. Three dilutions of each serum sample were made as follows: (1) a 1:4 dilution in broth; (2) a 1:20 dilution in 25% normal rabbit (or pooled human) serum broth; (3) a 1:120 dilution in 25% normal serum broth; and (4) a 1:600 dilution in 25% normal serum broth. If it was expected that the concentration of terramycin per ml of serum may be low, undiluted serum likewise was tested. When dilutions were prepared in this manner a concentration of 25% serum existed in all except a portion of the undiluted serum series.†

For each dilution of serum, a series of 6

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1. Hobby, G. L., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 503.

* The nature of the 2 forms of terramycin sodium salt has been discussed previously.(1)

2. Tompsett, R., *et al.*, *J. Bact.*, 1947, v53, 581.

† No correction has been made for the high concentration of serum in these tubes. It is assumed that the inhibition of the growth of *Streptococcus hemolyticus* in these tubes is indicative of the presence of terramycin; it is recognized, however, that the concentration in the serum cannot be measured accurately by the method used herein.

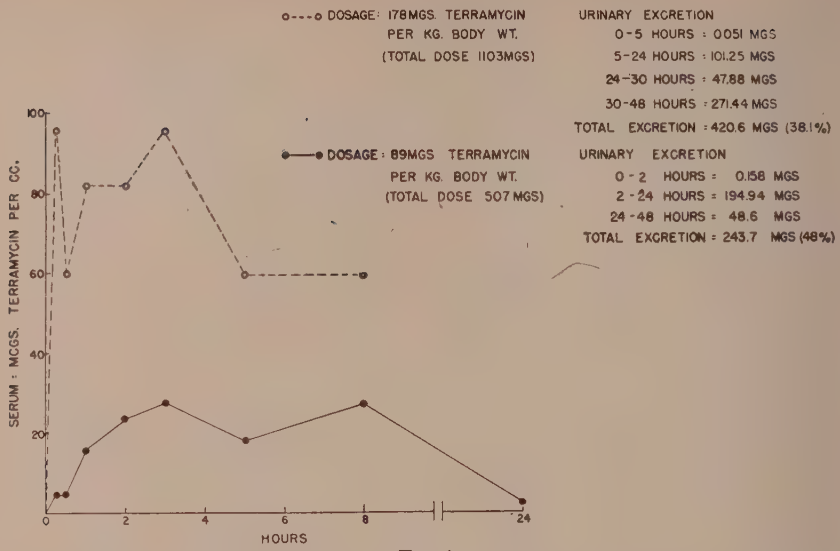

FIG. 1.

The absorption and excretion of terramycin following single intramuscular injections of terramycin sodium salt in dogs.

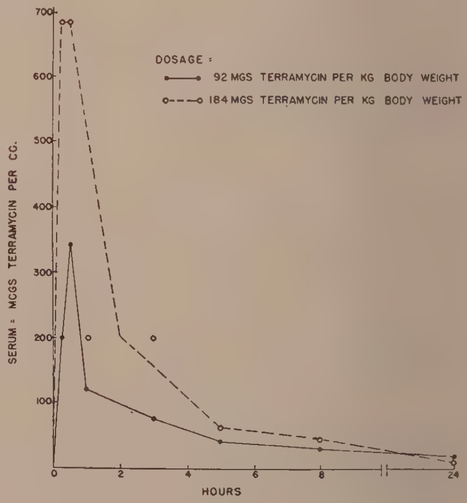

FIG. 2.

Concentrations of terramycin in serum following single intravenous injections of terramycin sodium salt in dogs.

tubes were used, containing 0.2 ml, 0.3 ml, 0.4 ml, 0.5 ml, 0.6 ml, and 0.8 ml of the diluted serum. The amount of fluid per tube was adjusted to a volume of 0.8 ml by the addition of 25% serum broth. Two-tenths ml of diluted culture was added to each tube and mixed well. *Streptococcus hemolyticus*, strain C203Mv, was used throughout; a $10^{-5}$ dilution of a 15-hour blood broth culture gave uniform and satisfactory results. All tests were incubated at 37°C for 18 to 24 hours. The end point was accepted as the least amount of serum capable of inhibiting the growth of the organism as evidenced by absence of gross turbidity at the end of the incubation period. The concentration of terramycin in urine was determined by a modification of this procedure. Plain broth was used in place of serum broth for all dilutions throughout the test. Higher dilutions were frequently necessary (1:1800, 1:5400, etc.). Incubation was carried out at 37°C for a period of 24 to 30 hours or longer, depending upon the rate at which the culture grows in the urine. As above, the end point was accepted as the least amont of urine capable of inhibiting growth of the test organism as evidenced by absence of gross turbidity.

Crystalline terramycin was used as standard[‡] for both serum and urine assays. Concentrations of 2.0, 1.0, 0.5, and 0.1 $\mu$g of terramycin per ml of broth[§] gave satisfactory results. The sensitivity of the test organism

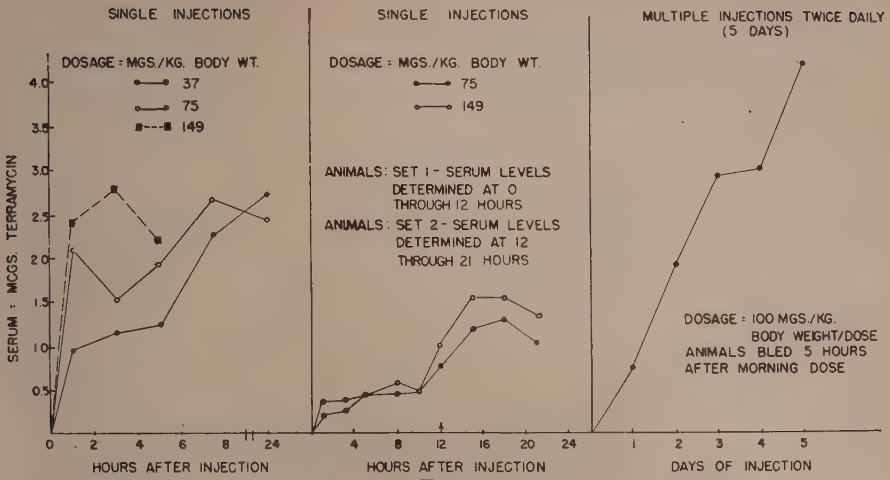

FIG. 3.

Concentrations of terramycin in serum following intramuscular administration in rabbits. (Average of 3 to 8 animals per dosage level.)

(C203Mv) to crystalline terramycin under the conditions of this test was generally 0.1 to 0.15 μg per ml.

*Results.* The results of a limited series of observations on the absorption and excretion of terramycin in rabbits and dogs are illustrated in Fig. 1 to 5.

*Dogs.* Following the intramuscular injection of the sodium salt of terramycin to dogs, in dosages of 89 to 178 mg per kg, terramycin was absorbed rapidly into the circulatory system. Significant concentrations were detected in the serum of injected animals within 15 to 30 minutes and peak concentrations were reached one quarter to three hours after injection. The rate at which the concentration in the blood stream reached its maximum varied with the dosage administered. Following intramuscular injection of a dosage equivalent to 89 mg per kg body weight, terramycin was demonstrated in the blood stream for a period of at least 24 hours. After intramuscular injection of 89 and 178 mg of pure terramycin per kg body weight of dogs terramycin appeared in the urine in biologically active form within 2 hours. Large quantities were recovered as late as 30 to 48 hours after administration. In the small series of dogs tested, the total excretion ranged from 38 to 48% of the administered dose. After intravenous injection in dogs, maximum concentrations of terramycin were detected in serum within 15 to 30 minutes. This was followed by a rapid decrease in the concentration present in the serum, and approximately 70% of the total dose administered was removed from the blood stream within 1 to 2 hours after injection. Low concentrations were detected, however, in the serum for as long as 24 hours after intravenous administration.

*Rabbits.* Extensive studies were carried out in rabbits to compare the rate of absorption of orally and parenterally administered terramycin and to determine differences, if

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‡ All studies reported herein were carried out using, as standard, pure terramycin (amphoteric) to which a potency of 1000 μg per mg had been assigned. This was a dihydrate compound. The current master standard is an anhydrous preparation of terramycin having a potency established at 1000 μg per mg; the working standard now in use is terramycin dihydrate having a designated potency of 925 μg per mg.

§ In all instances, standard was dissolved in serum broth and subsequently Seitz filtered to ensure sterility. As discussed in a previous communication(1), filtration of small volumes of dilute aqueous solutions is not recommended.

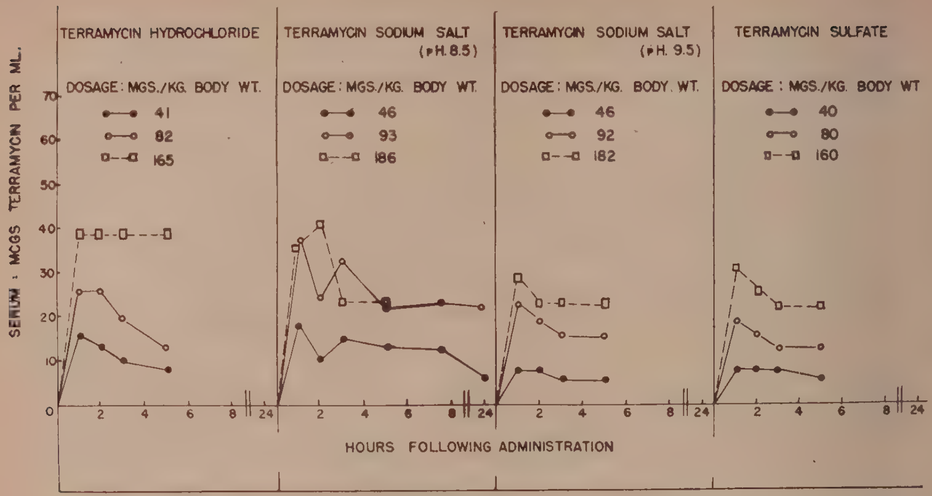

FIG. 4.

Concentrations of terramycin in serum following single intramuscular injection in rabbits. (Average of 3 to 8 animals per dosage level.)

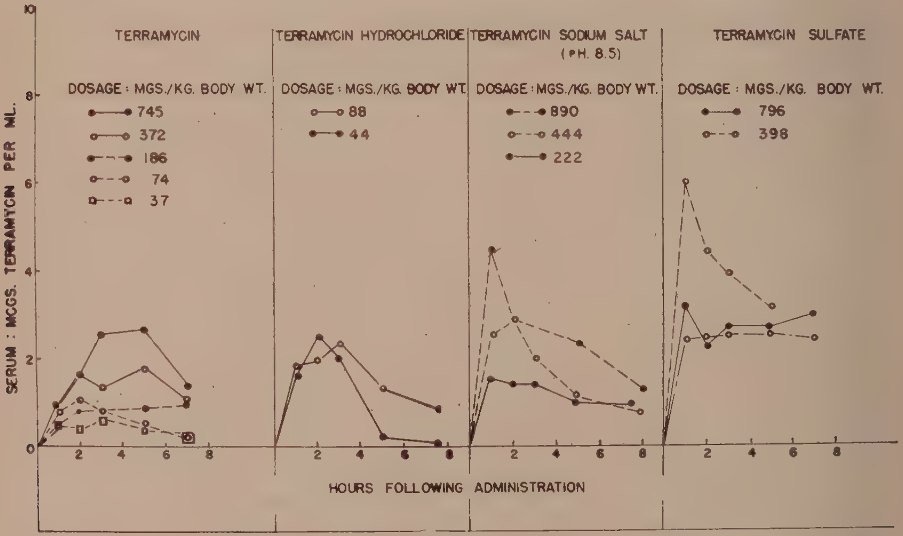

FIG. 5.

Concentrations of terramycin in serum following single doses administered orally in rabbits. (Average of 3 to 8 animals per dosage level.)

any, in the rates of absorption of terramycin and its salts. It is recognized that the rabbit is not the optimum animal for absorption studies. Large scale experiments carried out on the absorption of orally and parenterally administered penicillin indicated, however, that significant results may be obtained in this species of animal provided the tests are carried out under standardized conditions. To obtain comparable blood levels approximately 6 times as much penicillin is required in rabbits as in man; consequently, it is believed that the ratio of terramycin dosages may be comparably different. Following the intravenous administration of 46 mg of pure terramycin per kg body weight, the average concentration of terramycin detectable in the serum at one hour after administration was 24 μg per ml; an average of 5 μg per ml of serum was detectable at 5 hours after injection.

High concentrations of terramycin were observed in the serum of rabbits following the intramuscular administration of terramycin hydrochloride or terramycin sodium salt. The concentrations obtained following intramuscular administration of pure terramycin were low, indicating slow absorption which may have been due, at least in part, to its extremely low solubility in aqueous solutions. On repeated intramuscular injection of terramycin, a slight increase in the concentration of antibiotic in the serum was noted; the increase was not sufficient, however, to account for the dosage used. In all instances, terramycin sulfate gave irregular results. This may have been due to the rapid rate at which pure terramycin precipitates from solutions of terramycin sulfate. Absorption studies in a large series of rabbits have indicated that terramycin is absorbed readily from the gastro-intestinal tract. Following oral administration, high concentrations were detected in the blood within one hour. Although higher concentrations were attained in the serum of rabbits following administration of terramycin by the oral than by the intramuscular route, most satisfactory results were attained on oral administration by use of terramycin hydrochloride or sodium terramycin.

*Discussion and summary.* Studies on the absorption and excretion of terramycin in man will be reported by Werner and his associates(3) at an early date. The results obtained by these investigators agree, in general, with those reported herein. Terramycin and its salts are absorbed readily following either oral or parenteral administration. Terramycin in the amphoteric form is a highly insoluble compound. It is absorbed following oral or parenteral administration at an exceedingly slow rate. A comparative study of the chemotherapeutic action of terramycin and its salts in experimental infections in mice(1) have suggested that the slow absorption of this form of terramycin may be a valuable asset in the treatment of certain infections, provided a dosage form can be prepared which will permit its parenteral administration to humans. Studies are in progress, furthermore, to determine the extent to which this highly insoluble form of terramycin is excreted in the feces.

Most satisfactory results have been obtained to date on oral administration of terramycin hydrochloride, a highly soluble salt. The high concentrations of terramycin which may be detected in the serum of animals following oral administration suggests a parallelism between terramycin and aureomycin and chloramphenicol. In contrast to aureomycin and chloramphenicol, however, a high percentage of the terramycin administered is excreted in a biologically active form. Terramycin hydrochloride in aqueous solution has a pH of 1.5; its administration, parenterally in aqueous solution, is not feasible. Preliminary observations suggest, however, that the intravenous administration of this form of terramycin in an appropriate buffer should be possible.

The rate and degree of absorption of terramycin and its salts suggest that terramycin, a highly active antimicrobial agent, should be readily effective in the treatment of infections due to terramycin-sensitive microorganisms. Based on the experimental data available to date, terramycin offers promise as a chemotherapeutic agent for infections in man.

Received February 15, 1950, P.S.E.B.M., 1950, v73

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3. Werner, C., *et al.*, 1950, in press.

# Some Specific and Non-Specific Effects of Thiamine Deficiency in the Rat.* (17729)

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Deficiencies of the vitamin B-complex and of thiamine have been known to cause adrenal enlargement since first reported by McCarrison(1), while only recently has attention been directed to the thymus atrophy also present in these deficiency states(2,3). The possibility that these apparently unrelated phenomena could be manifestations of the same physiological process was not recognized until Deane and Shaw(4) demonstrated that the adrenal and thymus changes which occurred during the course of some vitamin deficiencies were typical of the general-adaptation-syndrome of Selye. The characteristic manifestations of this syndrome occur when an organism is subjected to non-specific stress and their development depends largely upon an increased functional activity of the pituitary-adrenal system. Thus, as adrenal hypertrophy and thymus atrophy are reliable indicators of adrenal-cortex hyperfunction, it seems apparent that certain vitamin deficiencies cause non-specific as well as specific alterations in the deficient animal.

As is well known the disorganization of carbohydrate metabolism is the biochemical effect of thiamine deficiency and other changes resulting from the avitaminosis may be secondary to the specific metabolic derangements *i.e.*, non-specific. Consequently this study was undertaken to establish whether the major anatomic manifestations of thiamine deficiency are specific or non-specific in nature.

*Materials and Methods.* Fourty-four male, hooded rats between 40-50 g in weight were divided into 3 groups and treated as follows: Group I served as absolute controls, the rats being allowed to eat the complete diet *ad lib.* The animals of Group II were fed the complete diet in sufficient quantity to maintain their weights essentially similar to the rats of Group III which received a thiamine deficient diet *ad lib.* Composition of the complete diet used in this experiment was as follows: Casein (vitamin free) 18%, Carbohydrate (1 part sucrose to 1 part starch) 71%, Fat (Shortening) 6%, Mineral Mixture (Steenbock) 4%, Cod Liver Oil 1%. A vitamin B mixture of the following composition was administered daily to each rat *per os*: Thiamine 40 μg, calcium pantothenate 250 μg, biotin 0.2 μg, P-aminobenzoic acid 250 μg, pteroylglutamic (folic) acid 10 μg, choline chloride 5 mg, inositol 2 mg. Thiamine was omitted from the vitamin mixture administered to the rats of Group III. Ten mg of α-tocopherol acetate was given to each rat orally every 10 days. Special cages were used to prevent coprophagy and the rats of Groups II and III were individually caged. All rats were weighed daily in order to rigidly control the food intake of Group II. After 36 days, the majority of the thiamine deficient rats showed signs of polyneuritis and disturbances of equilibrium. The experiment was then terminated, the animals being killed by decapitation. The organs to be studied were removed immediately and placed in Suza fixative for hematoxylin and eosin sections, while the adrenals were fixed in 10% neutral formalin and stained for fat with Sudan IV. Ascorbic acid determinations (method of Carruthers as modified by Moya *et al.*(5)) were made on the adrenals of 6 rats from each

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* This work was supported by a research grant from the Commonwealth Fund of New York.

† This work was done while the author was holder of a Research Fellowship from the Hoffmann-LaRoche Co., Nutley, N. J.

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2. Stoerk, H. C., and Zucker, T. F., PROC. SOC. EXP. BIOL. AND MED., 1944, v56, 151.

3. Christensen, K., and Griffith, W. H., *Endocrinology*, 1942, v30, 574.

4. Deane, H. W., and Shaw, J. H., *J. Nutrition*, 1947, v34, 1.

5. Moya, F., Prado, J. L., Rodriguez, R., Savard, K., and Selye, H., *Endocrinology*, 1948, v42, 223.

group. All organs were weighed on the chainomatic balance prior to sectioning.

*Observations.* A summary of the experimental observations is presented in Table I. It can be seen that the rats receiving the thiamine deficient ration (Group III) gained weight initially (maximum attained on the 14th day of the experiment) followed by a progressive decline until, by the end of the experimental period, the initial weight was almost reached. The body weight changes of Group II paralleled those of Group III thus adequately controlling the inanition factor involved in this type of experiment. Since the growth of Group I was satisfactory the composition of the synthetic diet seems nutritionally adequate.

There is a significant degree of adrenal hypertrophy and thymus involution in both the inanition (Group II) and thiamine deficient rats (Group III) compared to the normal controls (Group I). These changes probably indicate increased activity of the pituitary-adrenal system. Since there is also a significantly greater adrenal hypertrophy and thymus atrophy in Group III than in Group II, P < .05 and < .01 respectively) it is apparent that thiamine deficiency itself can function as a stressing agent, although inanition caused by the anorexia, invariably present in the avitaminosis, undoubtedly contributes to the actual degree of non-specific stress. Both kidney and liver were smaller by actual weight in the deficient and paired weight control rats than in the normal controls (Group I). The corrected weights of these organs expressed in the table, however, indicate that both partial inanition and thiamine deficiency produced a relative renal hypertrophy. In contrast the paired weight group showed hepatic atrophy while the thiamine deficient group evidenced no change in liver weight. It should be noted that the renal enlargement produced by the deficiency is very significantly greater than that caused by the equal degree of inanition in Group II. Although a relative increase in cardiac weight was present in both Groups II and III, the increase in the thiamine deficient group was significantly greater than in the paired weight controls. This suggests an actual cardiac

TABLE I.
Experimental Data on Thiamine-Deficient Rats and Their Controls.

| Group | No. rats | Body wt in g | | | Organ wts expressed in mg/100 g body wt. | | | | | | | | | | Adrenal ascorbic acid† mg/100 g tissue |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Initial | Highest | Final | Adrenal* | Thymus | Kidney | Liver | Heart | Testes | Seminal Vesicles | Spleen | Brain | Pituitary | |
| I Normal Controls | 14 | 42±.4 | | 138±6 | 17.8±.4 | 213±11 | 954±37 | 6180±202 | 330±14 | 1647±36 | 168 ±11 | 582±37 | 1117±77 | 3.6±.16 | 299±21 |
| II Paired Weight Controls | 15 | 42±.4 | 71±3 | 55±2 | 42.9±2.6 | 53±5.1 | 1150±34 | 5423±230 | 380±4 | 1790±148 | 19.6±3.7 | 495±67 | 2321±67 | 4.7±.06 | 275±22 |
| III Thiamine Deficient | 15 | 43±.3 | 70±2 | 53±2 | 50.4±2.5 | 28±2.2 | 1793±86 | 6210±169 | 430±17 | 1902±106 | 20.0±1.3 | 413±39 | 2608±142 | 5.2±.25 | 212±18 |

* Values obtained from 7 rats.
† " " " 6 "

enlargement due to thiamine deficiency. The uncorrected weights of the testes and seminal vesicles showed developmental failure in both thiamine deficiency and chronic inanition groups. When the weights of these organs were corrected to 100 g of body weight the testicular atrophy seemed to be proportional to the retardation of growth while the seminal vesicle atrophy was apparently much more pronounced. This evidence could be interpreted to mean a greater pituitary secretion of follicle stimulating hormone (F.S.H.) than luteinizing hormone (L.H.). The spleen was equally atrophic in both the experimental and inanition control rats. The brain and pituitary did not change in actual weight which results in the relative increase in size shown by the corrected weights in the table.

Histologically, the adrenals of the thiamine deficient and paired weight control rats contained a greater amount of sudanophilic material than the normal controls. The adrenal content of lipid was greatest in thiamine deficiency, in distinct contrast to the ascorbic acid content which was lowest in this group. The involution of the thymus was more pronounced in the thiamine deficient group where the gland consisted almost entirely of connective tissue stroma. In the paired weight controls it was of almost normal structure although greatly reduced in size. Cross-section of the fixed kidneys from the thiamine deficient group showed an increase in cortical width with a well defined white band in the region corresponding to the inner cortex. No such zone was seen in the kidneys of the inanition or normal controls. Microscopically this zone showed some tubular and glomerular enlargement which probably caused the observed increase in width of the cortex. The kidneys of the inanition controls had no comparable changes; indeed the cortex was thinner glomeruli and tubules smaller than in thiamine deficiency. The liver of the normal and thiamine deficient rats had essentially the same microscopic appearance. The cells were large with vesicular nuclei and fluffy vacuolized cytoplasm. In contrast, the cells of the paired weight controls were small with compact nuclei and dense, homogeneous, eosinophilic cytoplasm.

Both testes and seminal vesicles of the thiamine deficient and paired weight groups showed developmental failure as compared to absolute controls. In the testes, there was only partial failure of spermatogenesis but a marked atrophy of the Leydig cells. The seminal vesicles were very atrophic with a low, infolded lining epithelium and only a slight amount of secretion. The microscopic structure of the heart, brain and spleen of both thiamine deficient and inanition control rats was normal.

*Discussion.* In a previous publication(6), it was shown that most of the gross anatomical changes studied in B-complex deficiency were manifestations of the general-adaptation-syndrome. Many of the characteristic findings of this syndrome, such as adrenal hypertrophy, thymus atrophy and failure of testicular and seminal vesicle development have been reported also for thiamine deficiency. Indeed Deane and Shaw(4) have followed the adrenal and thymus changes through all three stages of alarm, resistance and exhaustion. In the above experiment the activation of the pituitary-adrenal system in thiamine deficiency was greater than that seen in equal inanition controls. However, as the partial starvation itself produced evidence of increased adrenal activity, part of the picture of thiamine deficiency is therefore due to the anorexia induced by the avitaminosis. It is of interest to note that at this stage of severe thiamine deficiency the adrenal sudanophilic lipids were increased above that seen in the normal or paired weight controls, yet the ascorbic acid content was significantly decreased. Apparently, as the adrenal cortical activity became exhausted in extreme thiamine deficiency, the ascorbic acid content decreased before the lipids were altered. This is another instance, besides that previously reported(7), where there is a dissociation between the adrenal content of stainable lipids and ascorbic acid.

The renal hypertrophy reported in B-complex and thiamine deficiency is probably another manifestation of non-specific damage. Constantinides(8) in unpublished experi-

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6. Skelton, F. R., *Am. J. Physiol.*, in press.

ments has shown that even after a stress of 48 hours duration there is enlargement of the kidney, while Dugan(9) has observed a marked enlargement after prolonged exposure to cold. Jiménez Diaz *et al.*(10) have reported a negative nitrogen balance during severe thiamine deficiency and Sandberg, Perla and Holly(11) have demonstrated that the nitrogen excretion during the deficiency exceeds that in inanition controls by 28%. Thus the renal hypertrophy seen in this experiment is probably due to the increased excretion of nitrogen metabolites which result from the protein breakdown caused either by a direct damaging effect of thiamine deficiency, increased gluconeogenesis from protein caused by the increased corticoid secretion or a specific effect of the avitaminosis on protein metabolism.

The relatively slower loss of liver and heart weight in thiamine deficiency as compared to inanition controls suggests a specific effect of the vitamin deficiency on these organs. This is not inconceivable in the light of known effects of the avitaminosis on carbohydrate metabolism and cardiac physiology. The distinct tendency to splenic atrophy in thiamine deficiency is in contrast to the tendency to hypertrophy previously reported for B-complex deficiency(6). Likewise the microscopic appearance of the spleen is strikingly different in thiamine deficiency than in B-complex avitaminosis. In the former the germinal centres of the follicles were of normal appearance while in the latter they have lost all sign of proliferative activity. In both experiments the inanition control rats have had a normal appearance of the splenic lymphatic follicles. These observations suggest that in thiamine deficiency one of the B factors present in the diet maintained either directly or indirectly the normal lymphatic activity of the spleen while its absence in B-complex deficiency caused failure of lymphopoiesis in the germinal centers of the follicles.

In thiamine deficiency as in B-complex deficiency, the evidence suggests that the secretion of follicle stimulating hormone (F.S.H.) by the pituitary is greater than the secretion of luteinizing hormone (L.H.) so that the seminal vesicles become much more atrophic than the testes because the development of the former depends on androgen production which in turn is controlled by the pituitary secretion of L.H.

*Summary and conclusions.* A detailed study was made of the gross and microscopic organ changes in the thiamine deficient rat as compared to normal and paired weight controls. It was apparent that the deficiency produced the essential changes characteristic of non-specific stress such as adrenal hypertrophy, thymus involution and decreased adrenal ascorbic acid. Marked renal hypertrophy was also observed in the deficient rats and was considered to be due to the prolonged negative nitrogen balance known to be associated with thiamine deficiency. The reduced gonadotrophin and increased adrenocorticotrophine secretion as indicated by the developmental failure of the sex organs and hypertrophy of the adrenals illustrates the so-called "shift" of pituitary hormone production under these circumstances. In contrast to the non-specific changes above, thiamine deficiency showed specific effects on the liver and heart. The activity of the germinal centres of the splenic lymphatic follicles was normally maintained in contra-distinction to the failure of germinal activity previously observed in B-complex deficiency.

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# Influence of Pregnancy in Mice on the Course of Infection with Murine Poliomyelitis Virus. (17730)

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It is generally believed that a majority of human beings exposed to the virus of poliomyelitis contracts the disease in sub-clinical form, only a small minority developing the characteristic paralytic type of infection. Some workers have suggested that those who escape paralysis may be protected by an "autarceologic" type of host resistance, the exact nature of which has not yet been established. Physiological fluctuations in the individual's resistance to the disease are illustrated in the possible enhanced susceptibility of women during pregnancy which has been noted by many clinical observers. A number of authors have recently attempted to evaluate by empirical or statistical methods the increased risk of infection with poliomyelitis during pregnancy(1-7). Although records of more than 380 cases may be found in the medical literature, information of epidemiological value is frequently lacking and, in many instances, inconsistent. The geographical distribution of these cases includes 3 continents, North America, Europe and Australia, the great majority originating in the United States and in the Scandinavian countries. In time, they cover a period of observation extending over 48 years. The small number included in any single outbreak has been insufficient to form a basis for adequate statistical analysis. In all events, the paucity of the data, the incomplete recording of cases and the purely empirical approach have only added to the uncertainty. Nevertheless, a general opinion has been formed that pregnancy predisposes to paralytic poliomyelitis(1,2,6-10) and, more particularly, that the pregnant woman is prone to develop the disease in its more severe manifestations, especially during the final trimester of gestation(3,4,5,11,12). On the other hand, occasional observers maintain that pregnancy alters neither the susceptibility to the disease(13) nor its course(2,14-16). A belief that women during the first trimester of pregnancy may actually be somewhat more resistant to infection was based upon disproportionately small numbers of cases which had been reported for the earlier part of pregnancy(10).

Experimental exploration of this problem was undertaken by Weaver and Steiner(17) who infected groups of pregnant and non-pregnant cotton rats with the Armstrong-Lansing C-R strain of poliomyelitis virus. Since resistant animals were found only in the group

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which had been infected during the first trimester of gestation, it was concluded that early pregnancy represents a period of increased resistance. The experiment was so designed that only an increase of resistance could be demonstrated, precluding the detection of any possible enhancement of susceptibility during the later stages. The present work was initiated in order to study experimentally the influence of pregnancy, throughout its entire course, upon the susceptibility of the white mouse to infection with murine poliomyelitis virus.

*Experimental. The Virus.* The infectious agent used throughout was the Col. SK strain of murine poliomyelitis virus. Now approaching the 400th serial passage in white mice, the strain has maintained its high infectivity for rodents and cynomolgus monkeys when injected by neural or extraneural routes.

*The mice.* The experimental animal was the Swiss white mouse, Rockland strain. Female mice weighing from 24 to 26 g were examined daily to establish the oestrous cycle(18). Upon the appearance of pro-oestrus each mouse was placed overnight with a normal male mouse. When one half of the mice had been successfully mated, the other half was set aside, unmated, to serve as control animals. Thus, the control group consisted of non-pregnant female mice of comparable age, which would also have been of comparable weight had it not been for the changes associated with pregnancy. A smaller number of mice was permitted to come to term; these were infected during the first 5 days after parturition. The experiment was planned so as to study the susceptibility of the mouse for each day of pregnancy throughout the entire gestation period. Since it was impossible to obtain, at one time, 100 pregnant mice suitably distributed in respect to their periods of gestation, the pregnant mice were infected, along with their non-pregnant controls, in small groups at various stages of pregnancy.

*Infection.* The inoculum was prepared from freshly harvested brains of young mice sacrificed at the height of paralysis. The brains were triturated and suspended in physiological saline at a dilution of $10^{-1}$. After light centrifugation the supernatant was employed for further dilution. Orally infected mice received by gavage 0.1 ml of virus suspension at a concentration of $10^{-2}$, an infection which, it was estimated, would approximate 50% lethality in the control group of mice. Apart from a total of 211 mice which were infected by feeding the virus, 2 smaller groups, comprising 19 pregnant and 19 non-pregnant animals, were infected by the intravenous route, each mouse receiving via the tail vein 0.1 ml of virus diluted $10^{-5}$.

The mice were examined daily over a period of 3 weeks for the appearance of typical symptoms of paralysis or encephalitis. When mice were found dead and symptoms had not been observed, the viral cause of death was confirmed by subpassage of the brains in at least 3 young mice by the intraperitoneal route, such brains having previously been shown to be bacteriologically sterile when cultured upon ordinary laboratory media. In all, 40 mice (27% of those which died) failed to exhibit typical symptoms.

*Results. Infection by the Oral Route.* Of a total of 100 orally infected pregnant mice, 75 died of the infection, while in a group of 100 non-pregnant mice, 45 succumbed to the disease. When the gestation period is divided into approximate quarters of 5 days each, the following mortality rates are observed (Table I): first quarter, 52%; second quarter, 72%; third quarter, 85%; fourth quarter, 88%. Eleven mice inoculated during the first 5 days post partum had a mortality rate of 46%. The incubation period of the disease is defined as the interval between the day of inoculation and the day upon which unequivocal symptoms first appeared, or, in the absence of symptoms, the day of death. The average incubation period in the 75 pregnant mice was 5.4 days, whereas in the 45 control animals it was 6.1 days. For the 4 quarters of pregnancy the respective figures were 6.7, 4.9, 5.3 and 5.5 days. The five mice of the post partum group had a mean incubation period of 6.7 days.

*Infection by the Intravenous Route.* Of

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18. Roscoe B. Jackson Memorial Laboratory, *The Biology of the Laboratory Mouse*, G. D. Snell, ed., Blakiston, Philadelphia, 1941.

TABLE I.
Mortality Among Pregnant and Non-pregnant Mice Inoculated with Col. SK Virus.

| | Oral inoculation of 0.1 cc virus diluted $10^{-2}$ | | | Intravenous inoculation of 0.1 cc virus diluted $10^{-5}$ | | |
|---|---|---|---|---|---|---|
| Time of inoculation | Mortality | % | Avg incubation time, days | Mortality | % | Avg incubation time, days |
| During gestation: | | | | | | |
| Days 1 to 5 | 11/21*§ | (52) | 6.7 | † | | |
| ” 6 to 10 | 21/29 | (72) | 4.9 | 5/ 8* | (63) | 7.4 |
| ” 11 ” 15 | 28/33 | (85) | 5.3 | ‡ | | |
| ” 16 ” 19 | 15/17 | (88) | 5.5 | 10/11 | (91) | 4.8 |
| Total: | | | | | | |
| All pregnant mice | 75/100 | (75) | 5.4 | 15/19 | (79) | 5.7 |
| During first 5 days post partum | 5/11 | (46) | 6.7 | | | |
| Normal non-pregnant female mice | 45/100 | (45) | 6.1 | 10/19 | (53) | 6.1 |

* Denominator: No. of mice inoculated; numerator: No. of mice died.

† Animals from first 10 days of pregnancy pooled.

‡ Animals from last 9 days of pregnancy pooled.

§ During first 3 days of pregnancy, 11 mice inoculated, of which 4 died (36%).

19 pregnant mice inoculated by the intravenous route, 15 (79%) died with an average incubation period of 5.7 days; 10 (53%) of 19 non-pregnant mice died with an incubation period of 6.1 days. Because of the small number of animals used in this part of the experiment, composite values for only the 2 halves of the gestation period are computed. Thus, 5 (63%) of the 8 mice infected on or before the 10th day died, whereas 10 (91%) of the 11 mice inoculated after the 10th day succumbed. The mean incubation periods for the 2 groups were 7.4 and 4.8 days respectively.

*Discussion.* Judging from the observed mortality rates and average incubation periods, white mice develop a gradual increase in susceptibility to oral infection with Col. SK virus beginning with the 4th day of gestation. Maximal susceptibility is reached toward the end of pregnancy, since the mortality rate at that time was very nearly twice that observed for the group of non-pregnant mice. The low mortality rate (36%) for mice inoculated during the first 3 days of pregnancy may be taken to indicate that such mice are at least as resistant as the controls, and possibly more so. Therefore the results agree well with the earlier observations of Weaver and Steiner(17) on infection with the Lansing strain in pregnant cotton rats, *i.e.* that early gestation may represent a period of increased resistance.

Since young mice, similarly infected, almost invariably die of the disease, the increased susceptibility of the adult mouse during gestation suggests a return to the maximal susceptibility characteristic of youth, and a temporary loss of the non-specific type of resistance associated with age. The transient nature of the phenomenon is illustrated by the fact that mice which are infected soon after parturition have a mortality rate which approximates closely that of non-pregnant adult females. That susceptibility to poliomyelitis may, in some way, depend upon disturbances in the endocrine balance is not a new thought. Aycock(19), for instance studied the possibility that changes in estrogen levels may be responsible for the frequent occurrence of the disease among pregnant women. When castrated immature female monkeys were treated with estrogenic substance they were found to be more resistant than untreated castrates. In these experiments the animals had been infected by the intranasal route, and it was held that changes in the nasal mucosa, resulting from elevated estrogen levels in the treated monkeys, might have inhibited passage of the virus. On the

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19. Aycock, W. L., *Endocrinol.*, 1940, v27, 49.

other hand, the average urinary estrogen levels of chronic poliomyelitis patients were found to be higher than those observed in normal individuals. The conflicting and confusing results prompted Aycock to conclude that "endocrinopathy sought as a basis of autarceologic susceptibility does not lie in a simple deficiency in elaboration of estrogenic substance, but rather in some discrepancy in its economy." A similar conflict has been noted in the case of the pregnant woman(3) and it has been suggested that fluctuations in resistance during gestation may depend upon variations in relative dominance of the manifold endocrine constituents rather than upon quantitative changes of any individual hormone in the system(20). However, the argument in favor of a protective function of chorionic gonadotropin in early pregnancy would appear to stand upon less conflicting evidence contributed by other investigators(17,21).

More recently, the influence of various endocrine substances was studied anew by Anderson and Bolin(22) in mice orally infected with the MM strain of murine poliomyelitis virus. While stilbestrol or progesterone treatment afforded a high order of protection, mice treated with t-propionate (presumably testosterone-propionate) were only partially protected, and those receiving desoxycorticosterone-acetate were as susceptible as the untreated animals. Again, it was suggested that the protective effects might have been due to alterations in the permeability of mucous surfaces preventing entry of the virus. In our work, however, there was no significant difference in the results which could be ascribed to the route of inoculation, whether oral or intravenous. Thus, our own observations serve to emphasize once more the possibility that the state of pregnancy may be associated with a systemic increase in susceptibility to poliomyelitis, irrespective of any changes that may exist at the portal of entry.

*Conclusions.* Pregnant mice inoculated with the Col. SK strain of murine poliomyelitis virus by the oral route showed a definite increase in susceptibility to infection as compared with non-pregnant female control animals. Beginning with the 4th day of pregnancy, mortality increased progressively and reached, with the last 4 days of gestation, a rate almost twice that of the control group. Immediately after parturition there was a rapid return of resistance to the non-pregnant level. The mechanism responsible for the greater susceptibility of the pregnant mouse does not seem to depend wholly upon alterations in the permeability of the intestinal mucosa during gestation, since a smaller group of mice infected by the intravenous route exhibited a similar increase in susceptibility.

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20. Davis, W. D., and Silverstein, C. M., *U. S. Naval Med. Bull.*, 1947, v47, 910.

21. Jungeblut, C. W., Meyer, K., and Engle, E. T., *J. Immunol.*, 1934, v27, 43.

22. Anderson, J. A., and Bolin, V., *Endocrinol.*, 1946, v39, 67.

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## Further Observations on the "Animal Protein Factor." (17731)

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A previous publication(1) from this laboratory has shown that an "animal protein factor" supplement (Fraction 1) made from cultures of *Streptomyces aureofaciens* produced a growth response in chicks which was greater than the maximum growth response obtainable with vitamin $B_{12}$. Distillers' solubles, fish meal, fish solubles and dried brewers' yeast did not produce additional growth

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1. Stokstad, E. L. R., Jukes, T. H., Pierce, J. V., Page, A. C., Jr., and Franklin, A. L., *J. Biol. Chem.*, 1949, v180, 647.

TABLE I.
Composition of Diets.

| Ingredient | Diets 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Soybean meal, expeller process | 30 | 70 | — | — | — | — |
| Fish meal | — | — | 25 | — | — | — |
| Cottonseed meal | — | — | — | 35 | — | — |
| Peanut meal | — | — | — | — | 35 | — |
| Sodium proteinate | — | — | — | — | — | 25 |
| Yellow corn meal | 63 | 23 | 69 | 57.4 | 57.4 | — |
| Cerelose | — | — | — | — | — | 59.7 |
| Bone ash | 1.0 | 1.0 | — | 1.0 | 1.0 | 2.5 |
| Calcium carbonate | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | — |
| ” gluconate | — | — | — | — | — | 5.0 |
| Salt mixture* | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 1.4 |
| Sodium chloride | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.6 |
| Vit. mixture in glucose† | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Corn oil + vit. A, D, and E‡ | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 3.0 |
| Methionine (DL–) | 0.3 | 0.3 | 0.3 | 0.4 | 0.4 | 0.3 |
| Choline chloride | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Lysine (DL–) hydrochloride | — | — | — | 0.5 | 0.5 | — |
| Glycine | — | — | — | — | — | 1.0 |
| Cystine | — | — | — | — | — | 0.3 |

* The salt mixture had the following composition: $K_2HPO_4$ 300, $KH_2PO_4$ 225, $MgSO_4$ 125, $MnSO_4$ (anhydrous) 20, ferric citrate 25, $CuSO_4 \cdot 5H_2O$ 1.0, KI 0.3, zinc acetate 0.7, $Al_2SO_4 \cdot 18H_2O$ 0.8, cobalt acetate 0.2, nickel carbonate 0.1 (Total 715).

† The vit. mixture contributed the following per kg of diet: Thiamine HCl 10 mg, riboflavin 10 mg, niacinamide 50 mg, pyridoxine 10 mg, calcium pantothenate 50 mg, pteroylglutamic acid 2 mg, biotin 0.2 mg, 1-acetoxy-2-methyl-4-napthyl sodium phosphate (vit. K derivative) 5 mg.

‡ Vit. A acetate 1,500 U.S.P. units, vit. $D_3$ 200 A.O.A.C. units, mixed tocopherols 34 mg dissolved in 1.0 g corn oil.

above that obtained with vitamin $B_{12}$. Cunha *et al.*(2) noted that the "animal protein factor" supplement produced a marked response in pigs on a corn-peanut meal basal diet while a vitamin $B_{12}$ concentrate had little effect. McGinnis and co-workers reported(3) that on a 56% soybean diet, a liver extract containing vitamin $B_{12}$ produced no growth response in young turkeys while the "animal protein factor" supplement gave a marked growth increase. The growth-promoting effects of various supplements upon chicks receiving a basal diet containing 70% of soybean meal(1) and adequately supplied with vitamin $B_{12}$ were studied in the present investigation. It was found that either crystalline aureomycin hydrochloride or a culture of *S. aureofaciens* in which the aureomycin had been inactivated had growth-promoting effects.

*Methods.* Depleted chicks with a low reserve of the animal protein factor were obtained by hatching eggs from hens on Diet 1(1). The chicks (Barred Rock x New Hampshire) were placed immediately after hatching on the experimental diets. Two types of aureomycin fermentation products were used.* Fraction 1 was a dry mixture of the mycelium plus a variable amount of filter-aid. A concentrate of the whole fermented mash from which water had been removed by evaporation was also used. Vitamin $B_{12}$ was supplied either in the crystalline form or as a mixture of vit. $B_{12}$ and $B_{12b}$ containing more than 10 $\mu$g of the mixed vitamins per mg of organic solids. The alkaline extract of fraction 1 was made by heating 1 kg of this fraction with an excess of 0.5 N sodium hydroxide, neutralizing and concentrating.

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2. Cunha, T. J., Burnside, J. E., Buschman, D. M., Glasscock, R. S., Pearson, A. M., and Shealy, A. L., *Arch. Biochem.*, 1949, v23, 324.

3. McGinnis, J., Stephenson, E. L., Lavadie, B. T., Carver, J. S., Garibaldi, J. A., Ijichi, K., Snell, N. S., and Lewis, J. C., Paper presented at the Am. Chem. Soc. Meeting, Atlantic City, N. J., Sept. 1949.

* The aureomycin fermented mash and fraction 1 were kindly supplied by Dr. S. H. Babcock and Dr. A. S. Phelps, Chemical Production Section, Lederle Laboratories.

TABLE II.
Response Produced by Vit. $B_{12}$ and Other Supplements on Different Types of Basal Diets.

| Exp. No. | Type of basal ration and supplement per kg of diet | | Wt and survivors ( ) at 25 days, g |
|---|---|---|---|
| I. | Diet 2 (70% soybean meal) | 30 $\mu$g $B_{12}$ | 281 (18)[*] |
| | | 30 " " + 15 g fraction No. 1 | 360 (12) |
| | | 30 " " + 30 ml conc. mash No. 2 | 368 (10) |
| | Diet 3 (25% fish meal) | 30 " " | 295 (10) |
| | | 30 " " + 15 g fraction No. 1 | 346 (11) |
| II. | Diet 1 (30% soybean meal) | $B_{12}$ injection[†] | 247 (12) |
| | | " " + 50 g dried whey | 242 (11) |
| | | " " + 4 g fraction No. 1 | 286 (12) |
| | Diet 2 (70% soybean meal) | " " | 260 (11) |
| | | " " + 8 g fraction No. 1 | 292 (11) |
| | Diet 4 (35% cottonseed meal) | None | 182 (12) |
| | | $B_{12}$ injection | 185 (11) |
| | | " " + 4 g fraction No. 1 | 216 (11) |
| | Diet 5 (35% peanut meal) | None | 239 (11) |
| | | $B_{12}$ injection | 240 (12) |
| | | " " + 4 g fraction No. 1 | 288 (10) |

[*] 24 chicks used initially in this group, 12 used in all others.
[†] 4 $\mu$g intramuscularly per week.

This extract showed no aureomycin activity when assayed[†] with *Staphylococcus aureus* by the method of Dornbush and Pelcak(4).

*Results. Amount of vit. $B_{12}$ needed for maximum growth:* It was necessary to supply sufficient $B_{12}$ so that any additional amounts of $B_{12}$ in the supplements to be tested did not produce an additional growth response. A number of experiments were made to determine the requirements for vitamin $B_{12}$. When given in the diet the requirement varied from 15 to 30 $\mu$g per kg of diet. Intramuscular injection of vitamin $B_{12}$ was also used. About 0.4 $\mu$g injected once weekly has been found to meet the vitamin $B_{12}$ requirement of chicks(1). In the assays for the unknown growth factor, 30 $\mu$g of vitamin $B_{12}$ were added per kilo of diet or injections of 4 $\mu$g were given on the 1st, 7th and 15th days.

*Response obtained on different types of diet.* Basal diets containing various protein concentrates were used and their composition is shown in Table I. In diets 4 and 5, containing cottonseed meal and peanut meal, lysine was included to make the diets more adequate with respect to amino acids and thus to eliminate the possibllity of the production of growth responses by any lysine which might have been present in the supplements being tested. The effects of vitamin $B_{12}$ with or without aureomycin fermentation fractions were tested with chicks and the results are shown in Table II. These fermentation fractions produced responses in chicks on diets containing either 30 or 70% soybean meal. It was found that vitamin $B_{12}$ produced no response when added to the diets containing fish meal, cottonseed meal and peanut meal. However, the aureomycin fractions produced a growth response when added to any of the diets and the percentage increase in growth over that obtained with the basal diets was approximately the same for all types of diets. The results with fish meal were of interest in that they indicated that fish meal did not furnish effective amounts of the unknown growth factor. Dried whey produced no growth response when added to diet 1 in the presence of vitamin $B_{12}$.

*Response to Crystalline Aureomycin.* Chemical fractionation of the aureomycin mash revealed some similarities between the distribution of aureomycin and that of the growth factor. Aureomycin hydrochloride which had been repeatedly recrystallized[‡] was

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[†] Assays for aureomycin were made through the courtesy of Miss Frances L. Clapp and Miss Frances Bingham, Lederle Laboratories.

4. Dornbush, A. C., and Pelcak, E. J., *Ann. N. Y. Acad. of Sci.*, 1948, v51, 218.

[‡] This sample of specially purified aureomycin was supplied through the courtesy of Dr. R. Winterbottom, Lederle Laboratories.

TABLE III.
Growth Stimulation Produced in Chicks by Various Supplements in Presence of Vitamin $B_{12}$.

| | | | Avg wt and No. of survivors ( ) Experiment No. | | | | |
|---|---|---|---|---|---|---|---|
| Supplement per kg of diet 2 | | | III* 21 days | IV* 25 days | V* 21 days | VI† 25 days | VII* 21 days |
| None | | | 248 (12) | 249 (11) | 207 (11) | 246 ( 5) | 221 (11) |
| " | | | | 268 (11) | | 269 ( 7) | 223 (12) |
| Concentrated aureomycin mash | 2.5 | ml | | | 247 (11) | | |
| " " " | 10 | " | | | 249 (11) | | |
| " " " | 30 | " | 288 (12) | 298 (11) | | | 273 (12) |
| " " " | 30 | " | | | | | 294 (11) |
| Aureomycin HCl | 25 | mg | | | 235 (11) | | |
| " " | 60 | " | 272 (11) | | | | |
| " " | 100 | " | | 309 (11) | 242 (11) | | 270 (11) |
| Fraction 1 | 5 | g | | | | 337 (12) | |
| " " | 15 | " | | | | 343 (12) | |
| Alkaline extract of 10 g, Fraction 1 | | | | | | 329 (12) | |
| " " " 30 " " " | | | | | | 342 (12) | |
| Streptomycin, $CaCl_2$ complex | 100 | mg | | | | | 255 (12) |
| " " " | 300 | " | | | | | 266 (12) |
| Succinylsulfathiazole | 10 | g | | | 228 (10) | | |
| 3-nitro-4-hydroxy-phenyl-arsonic acid | 50 | mg | | | | | 265 (12) |
| " " " " " " " | 100 | " | | | | | 241 (12) |

* 4 μg vit. $B_{12}$ injected weekly.
† 30 " " " added per kg of diet.

found to produce a growth response. Some fractions were obtained from fermented mash which were almost devoid of aureomycin but which still retained growth activity. Furthermore, an extract prepared by heating fraction with 0.5 N NaOH gave a growth response in chicks although all the aureomycin had been destroyed by the treatment. Aureomycin fermented whole mash was also heated with sodium hydroxide without destroying the growth-promoting activity, although all the aureomycin had been inactivated. On the basis of a large number of experiments crystalline aureomycin did not appear to give uniformly as great a response as the aureomycin whole mash. It seems unlikely that all the growth-promoting activity of the fermented mash can be accounted for on the basis of its aureomycin content.

Since the addition of succinylsulfathiazole and streptomycin to a purified diet have been reported to produce a growth-promoting effect(5), these compounds were tested on diet 2. The results which are presented in Table III, show that succinylsulfathiazole produced a response which was not as large as that produced by aureomycin hydrochloride or aureomycin mash. A level of 100 mg of streptomycin gave a partial increase while significant responses with aureomycin were obtained with levels as low as 25 mg per kg of diet. A response was obtained with 300 mg of streptomycin. This is in agreement with the results of Moore and co-workers(5) who found that 500,000 units of streptomycin, (*ca* 500 mg) per kg of a purified diet gave a better response than 200,000 units, (*ca* 200 mg).

Morehouse and Mayfield(6) and Morehouse(7) have described the growth stimulation produced in chickens and turkeys by 3-nitro-4-hydroxyphenylarsonic acid. Bird and co-workers(8,9) have also presented evidence on the growth-promoting activity of various phenylarsonic acid derivatives with

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5. Moore, P. R., Evenson, A., Luckey, T. D., McCoy, E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1946, v165, 437.

6. Morehouse, N. F., and Mayfield, O. J., *J. Parasitol.*, 1946, v32, 20.

7. Morehouse, N. F., *Poultry Science*, 1949, v28, 375.

8. Bird, H. R., Groschke, A. C., and Rubin, M., *Fed. Proc.*, 1948, v7, 283.

9. Bird, H. R., Groschke, A. C., and Rubin, M., *J. Nutrition*, 1949, v37, 215.

TABLE IV.
Effect of Various Supplements on "Sodium-Proteinate" Diet—Basal Diet No. 6.
(12 chicks per group).

| Vit. $B_{12}$ per kg diet, μg | Supplement per kg diet | Wt and survivors ( ) 25 days, g |
|---|---|---|
| None | None | 173 ( 9) |
| 50 | " | 229 (11) |
| 50 | Brewer's yeast 75 g | 250 (12) |
| 50 | Fish meal 50 g | 234 (12) |
| 50 | Aureomycin fermented mash 30 ml | 330 (12) |

chicks. In the experiments summarized in Table III, growth responses were obtained with 3-nitro-4-hydroxyphenylarsonic acid although no level of this compound which was used produced gains as large as that obtainable with aureomycin mash.

*Growth response produced on soybean protein diet.* Carlson *et al.*(10) have reported that yeast contained an unidentified growth factor, as evidenced by the response produced on a diet containing "alpha-protein" and supplemented with 11.6 μg of vitamin $B_{12}$ per kg of diet. With Diet No. 6, similar to that used by Carlson(10) except that "sodium proteinate"§ was used in place of "alpha-protein," we obtained only a slight response with yeast and no response with fish meal when 50 μg of vitamin $B_{12}$ were added per kg of basal ration. These results, which are shown in Table IV, also show that aureomycin fermented mash produced a gain of 53% above that produced on the basal diet supplemented with $B_{12}$. Thus, it is apparent that the nature of the growth response produced by aureomycin fermentation products is distinct from that described by Carlson and co-workers(10). The ineffectiveness of whey (Table II) appears to distinguish the growth response described here from that produced by the "whey factor" described by Hill(11).

*Discussion.* The growth stimulation which was observed in chicks on aureomycin fermentation materials can be attributed, at least in part, to aureomycin. Unpublished experiments(12) indicate that aureomycin produces growth responses in pigs and turkeys under conditions(2,3) in which these species were reported to respond to fraction 1.

The possibility that the growth produced by aureomycin is related to its antibiotic activity is raised by our experiments. The fact that growth responses have been obtained by such widely differing types of compounds as succinylsulfathiazole, streptomycin and substituted phenylarsonic acids supports this view. Harned *et al.*(13) in a single experiment with two groups of 6 chicks reported that the addition of aureomycin increased the growth rate and attributed the effect to the elimination of some infection. No infections were noted in the present investigation.

*Summary.* 1. Fermentation products of *Streptomyces aureofaciens* were found to promote growth in depleted chicks on various diets which were adequately supplied with vitamin $B_{12}$.

2. Growth responses in chicks on a corn-soybean diet were also produced by crystalline aureomycin hydrochloride and by cultures in which the aureomycin, as measured by antibiotic potency, was destroyed by alkaline hydrolysis.

3. Responses were also obtained with succinylsulfathiazole, streptomycin and 3-nitro-4-hydroxyphenylarsonic acid, but these substances appeared less potent than aureomycin. No responses were obtained with dried whey or dried brewer's yeast.

*Addendum.* Following the submission of this manuscript, Dr. Paul Gyorgy kindly showed us a manuscript (Gyorgy, P., Stokes, J., Jr., Smith, W. H., and Goldblatt, H., *Transactions of Conference on Biological*

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10. Carlson, C. W., Miller, R. F., Peeler, H. T., Norris, L. C., and Heuser, G. F., *Poultry Science*, 1949, v28, 750.

§ A purified protein prepared from soybean meal and obtained through the courtesy of Archer-Daniels-Midland Company.

11. Hill, F. W., *Poultry Science*, 1948, v27, 536.

12. Stokstad, E. L. R., Jukes, T. H., Taylor, R. R., Cunha, T. J., Edwards, H. M., and Meadows, G. B., *Arch. Biochem.*, 1950, in press.

13. Harned, B. K., Cunningham, R. W., Clark, M. C., Cosgrove, R., Hine, C. R., McCauley, W. J., Stokey, E., Vessey, R. E., Yuda, N. N., and SubbaRow, Y., *Ann. N. Y. Acad. Sci.*, 1949, v51, 182.

*Antioxidants,* sponsored by the Macy Foundation, November, 1949) which described a beneficial effect exerted by aureomycin on growth and the prevention of dietary hepatic necrosis in rats. The assumption was made that aureomycin might act through the suppression of the intestinal flora.

The assistance of Vincent Stryeski, Alfred Plattner and Joseph Lang in the care of the animals is gratefully acknowledged.

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## An Improved Vertical Cylinder Oxygenator.[*] (17732)

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*From the Department of Surgical Research, Jefferson Medical College of Philadelphia.*

Prolonged interruption of blood flow to the heart and lungs followed by survival of the animals has been accomplished(1). The blood was oxygenated extracorporeally by blowing oxygen over a blood film upon the inner surface of a revolving vertical metal cylinder of 0.23 sq. meter surface area. A larger oxygenator of this type was described in detail(2). Recently, a still larger vertical cylinder, 79 cm in height, and 30 cm in internal diameter, has been constructed which differs from those previously described only in having a larger surface area, 0.71 sq. meter. It was thought that if the blood flow on the smooth inner surface of the cylinder could be made turbulent, the capacity of the apparatus to introduce oxygen could be increased. The basic apparatus was therefore modified by closely adapting a 16-mesh, 26-gauge wire screen to the cylinder's entire inner surface.

Data showing the relative oxygenating efficiency with and without the screen are shown in Table I. These values for oxygen introduced by a single passage of blood over the oxygenating surface were chosen from a number of determinations for the similarity of their initial venous oxygen saturations.

The maximum amount of oxygen which

TABLE I.
Oxygenating Capacity—Screened and Smooth Surfaces.

| Observation No. | Type of surface | $O_2$ content vol. % |  | $O_2$ capacity Vol. % | % saturation |  | Bld flow ml/min. | cc $O_2$ added per 100 ml blood | Total cc $O_2$ added per min. |
|---|---|---|---|---|---|---|---|---|---|
|  |  | Venous | Arterial |  | Venous | Arterial |  |  |  |
| 1 | Smooth | 8.9 | 14.8 | 21.9 | 40.6 | 67.4 | 1080 | 5.8 | 63.1 |
| 2 | Screen | 8.7 | 21.9 | 21.8 | 40.1 | 100.9 | 856 | 13.2 | 113.3 |
| 3 | Smooth | 8.9 | 15.5 | 21.9 | 40.6 | 70.8 | 1080 | 6.6 | 71.3 |
| 4 | Screen | 8.7 | 21.4 | 21.8 | 40.1 | 98.5 | 1360 | 12.7 | 172.2 |
| 5 | Smooth | 9.2 | 16.5 | 18.9 | 48.2 | 87.0 | 510 | 7.3 | 37.0 |
| 6 | Screen | 8.2 | 14.8 | 17.2 | 47.7 | 86.2 | 8200 | 6.6 | 542.8 |
| 7 | Smooth | 10.8 | 15.1 | 18.4 | 58.7 | 82.1 | 1125 | 4.3 | 48.4 |
| 8 | Screen | 9.8 | 16.0 | 18.1 | 54.1 | 88.5 | 2000 | 6.2 | 124.0 |
| 9 | Smooth | 10.8 | 15.6 | 18.4 | 58.7 | 84.8 | 1000 | 4.8 | 48.0 |
| 10 | Screen | 9.8 | 14.9 | 18.1 | 54.1 | 82.3 | 7160 | 5.1 | 365.0 |
| 11 | Smooth | 11.1 | 14.7 | 18.4 | 60.3 | 79.8 | 1210 | 3.6 | 43.5 |
| 12 | Screen | 11.2 | 14.2 | 17.2 | 65.2 | 82.5 | 7160 | 3.0 | 215.0 |
| 13 | Smooth | 11.1 | 16.0 | 18.4 | 60.3 | 86.9 | 1000 | 4.9 | 49.0 |
| 14 | Screen | 11.2 | 14.7 | 17.2 | 65.2 | 85.5 | 7160 | 3.5 | 251.0 |

Single passage over smooth and screen oxygenating surfaces at comparable initial oxygen saturation levels. Venous blood is blood before passage and arterial after passage.

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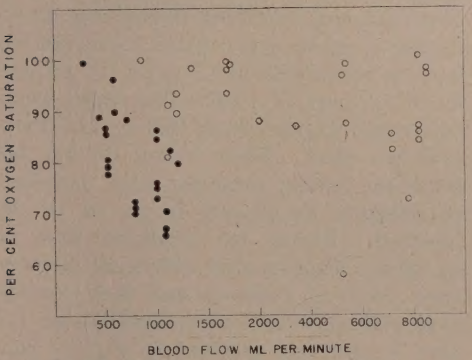
1. Gibbon, J. H., Jr., *Surg., Gynec. and Obst.,* 1939, v69, 602.

2. Gibbon, J. H., Jr., and Kraul, Charles W., *J. of Lab. and Clin. Med.,* 1941, v26, 1803.

could be added to the blood with the original apparatus was 71.3 cc per minute, with a change in oxygen saturation from 40.6% to 70.8% (Observation No. 3, Table I), at a blood flow rate of 1080 ml per minute. Since the addition of a screen, a maximum of 543 cc of oxygen per minute has been added to the blood with an increase in saturation from 47.4% to 86.2% at a blood flow rate of 8,200 ml per minute. With nearly identical venous oxygen saturations, 48.2 and 47.7%, the attainment of closely similar arterial saturations, 87.0 and 86.2% respectively, occurred at a flow rate of 510 ml per minute without the screen and at 8,200 ml per minute with the screen (Observations No. 5 and 6, Table I).



○ Screen surface. ● Smooth surface.

FIG. 1.

The % of oxygen saturation attained after single passage over screened and smooth oxygenating surfaces. Oxygen saturation before passage was approximately the same.

Prior to the introduction of the screen, as the blood flow rate was progressively increased above 1 liter per minute, the blood oxygen saturation progressively decreased. With the screen however, the flow rate can be increased 8 times this amount, with satisfactory blood oxygenation. This remarkable difference between the 2 methods in the flow rate at which blood can be adequately saturated with oxygen is illustrated in Fig. 1.

The efficiency of the vertical revolving cylinder for the oxygenation of blood has been increased 700 to 1000% by the addition of a screen. This modified apparatus has been used to supplant the cardiorespiratory function of 2 dogs for 25 and 37 minutes, followed by survival to date, 3 and 5 months respectively, without organic damage.

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## Failure of Cortisone or ACTH to Reduce Mortality in Irradiated Mice. (17733)

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The role of the adrenal cortex in the syndrome of irradiation illness is not well established(1). It seemed conceivable that cortisone (17-hydroxy-11-dehydrocorticosterone), or ACTH (adrenocorticotropic hormone), might reduce mortality in acutely irradiated animals. A group of experiments was designed to test this possibility. Because of the great current interest in the effect of these compounds in a variety of disease states a report of their failure to protect mice given a lethal amount of whole-body X-radiation appears to be appropriate.

Male mice of N.I.H. stock, kept on a diet of Purina chow, were caged 10 together from weaning to the time of irradiation when they were about 7 weeks old. They were then separated into comparable groups by litter and treated as shown in Fig. 1 and Table I. Ten mice were irradiated simultaneously, 5 each from the irradiated control and corresponding irradiated treated groups. Animals received 600 r whole-body radiation in the cortisone experiments, 500 r in the ACTH experiments,

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1. Patt, H. M., Swift, M. N., Tyree, E. B., and John, E. S., *Am. J. Physiol.*, 1947, v150, 480.

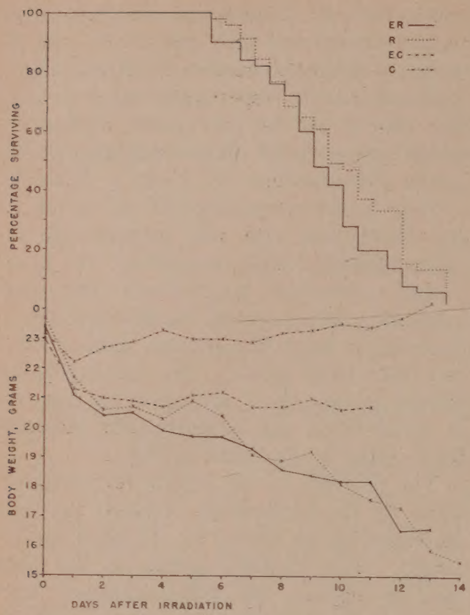

FIG. 1.

Death rate and weight changes in 50 mice given daily i.p. injections of 0.2 mg cortisone for 10 days, beginning 4 hr after irradiation with 600 r (ER) and in an equal number of irradiated littermate controls (R); weight changes in 15 injected controls (EC) and in controls not injected or irradiated (C).

with the following radiation factors: 170 Kv., 20 ma., 0.55 mm aluminum and 0.25 mm copper added filtration, 50 cm focal distance and rate 58 r per minute. After irradiation the mice were kept individually in suspended wire mesh cages.

Daily intraperitoneal injection of 0.2 mg of cortisone, begun a few hours after irradiation and continued for 10 days, was ineffectual in preventing death, increasing survival time, or reducing the severity of radiation effects on erythrocyte count, hemoglobin, reticulocytes, leucocyte count, or body weight. Death rate and weight changes are shown in Fig. 1. Untreated controls (C) show a transitory weight loss when transferred to individual cages. Cortisone controls (EC) lost about 2 g which they failed to recover by the 11th day. Cortisone controls, between the 7th and 11th day after beginning injections, showed a mean reduction in erythrocyte count of 1.4 million per mm$^3$, in hemoglobin of 3.3 g per 100 ml, and in reticulocytes of 1.2%. The mean leucocyte count dropped from 18.0 to 12.6 thousand per mm$^3$. These values do not fall below the normal range for mice, and their physiological significance is yet to be determined. Among the irradiated mice no significant hematological differences between those receiving cortisone and their controls were evident, reductions in both groups (15 mice in each, not included in Fig. 1) conforming to expectations for mice irradiated with 600 r, $LD_{100}$.

Cortisone given in 2 intraperitoneal injections of 0.2 mg each 21 hours and 3 hours prior to irradiation with 600 r did not significantly reduce mortality (RE, 20 mice, 95%; R, 20 mice, 100% mortality).

Subcutaneous injections of 0.25 mg of ACTH in 0.2 ml of saline begun a few hours after irradiation and given twice daily for 14 days post-irradiation were ineffectual in delaying or preventing death (Table I). In this experiment the $\chi^2$ value, 11.8, indicates that the effect of the compound was harmful,

TABLE I.
Percentage Mortality and Mean Survival Time of Irradiated Mice (500 r) Receiving ACTH Compared with Irradiated Controls.

| Group | Treatment | No. of mice | % dead | M.S.T., days |
|---|---|---|---|---|
| ACTH-C | No irradiation; ACTH as in R-ACTH-1 | 5 | 0 | — |
| R-ACTH-1 | 0.25 mg ACTH twice daily from 3 hr post-irradiation | 29 | 79.3 | 9.4 |
| R-1 | No inj.; mates to R-ACTH-1 | 29 | 31.0 | 11.6 |
| R-ACTH-2 | 0.5 mg ACTH 22 hr and 5 hr pre-irradiation | 19 | 63.5 | 10.6 |
| R-2 | No inj.; mates to R-ACTH-2 | 19 | 57.9 | 11.9 |

but the numbers are small and general conclusions are unwarranted. When ACTH was given in 2 injections prior to irradiation no effect was observed.

*Summary.* Intraperitoneal injections of cortisone, or subcutaneous injections of ACTH, failed to increase survival time or number of irradiated mice surviving.

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